NEUROHORMONAL CONTROL OF ADRENOCORTICOTROPHIN SYNTHESIS AND RELEASE

by

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TO MY FAMILY

ABSTRACT

The techniques of hypophysial portal blood collection and radioimmunoassay were used to examine the relationships between and the relative importance of corticotrophin-releasing hormone (CRH), vasopressin (AVP) and oxytocin (OT) in stimulating the secretion of adrenocorticotrophin (ACTH) in vivo. Factors affecting synthesis of pro-opiomelanocortin (POMC), the precursor for ACTH, were examined by measuring the content of POMC mRNA in the anterior pituitary gland by Northern blot analysis.

After adrenalectomy, there is an increase in the release of CRH and AVP into portal blood and an increase in the synthesis and secretion of ACTH. Dexamethasone injection decreased the release of AVP into portal blood collected 3h after the injection, but had no effect on the release of CRH. The responsiveness of the pituitary to CRH, however, was reduced by dexamethasone injection. These results suggest that glucocorticoids act both at the hypothalamus and at the pituitary gland to control ACTH secretion. Increased secretion and synthesis of ACTH was found to occur after a 5 min period of ether stress. Ether stress resulted in a prolonged increase in plasma ACTH and corticosterone concentrations which was followed after 6h by an increase in the content of POMC mRNA in the anterior pituitary gland.

Electrical stimulation of the paraventricular nuclei (PVN) significantly increased the release of CRH but not AVP into portal blood. The increase in CRH release was correlated with a significant increase in the peripheral plasma concentration of ACTH and corticosterone in rats in which the pituitary stalk was intact. However, PVN stimulation had no effect on the content of POMC mRNA in the anterior pituitary gland. Stimulation of the amygdala and the hippocampus decreased the release of CRH but not AVP into portal blood. These results suggest that the PVN is an important central node involved in the regulation of ACTH and thereby glucocorticoid release. Electrical stimulation of the median eminence did not increase the release of CRH, AVP or OT into portal blood possibly due to inhibiting effects which might involve antidromic and/or recurrent colateral stimulation. Infusion of naloxone before electrical stimulation of the median eminence did not increase the release of AVP or of OT into portal blood.

The content of immunoreactive AVP in hypophysial portal blood from homozygous Brattleboro rats was approximately 50% of that in Long Evans rats. Analysis by high performance liquid chromatography showed that the AVP in portal blood from Brattleboro rat co-eluted with synthetic AVP. There was no significant difference in the CRH or OT concentrations in portal blood between Long Evans and Brattleboro rats. ACTH and corticosterone concentrations in plasma were lower in heterozygous Brattleboro rats compared with those in Long Evans rats. The POMC mRNA content in the anterior pituitary gland in Brattleboro rats is 2-fold higher than in Long Evans rats. The presence of AVP in the portal blood of homozygous Brattleboro rats suggest that there may be some processing and release of AVP from the defective AVP-neurophysin-glycopeptide precursor in the external layer of the median eminence.

I declare that the studies presented in this thesis are the result of my own independent investigation with the exception of the histological assessment of the position of the stimulating electrodes which was carried out in collaboration with Dr. I. Laszlo (Chapter 7) and the analysis of hypophysial portal blood by high performance liquid chromatography which was carried out by Dr. I.C.A.F. Robinson (Chapter 5).

This work has not been, and is not being concurrently submitted for candidature for any other degree.

Some of the results presented in this thesis have been published as follows:

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ABBREVIATIONS

ACTH	Adrenocorticotrophin
ARGG	Anti-rabbit gamma globulin
AVP	Arginine vasopressin
CLIP	Corticotrophin-like intermediate peptide
CRF	Corticotrophin releasing factor
CRH	Corticotrophin releasing hormone
dDAVP	[1-deamino-8-D-arginine] vasopressin
DNA	Deoxyribonucleic acid
GABA	γ Amino-n-butyric acid
GnRH	Gonadotrophin releasing hormone
5-HT	5-hydroxytryptamine
i.p.	intraperitoneally
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
β-LPH	β-lipotropin
γ-LPH	γ-lipotropin
α-MSH	α-melanocyte stimulating hormone
mRNA	Messenger ribonucleic acid
NIL	Neurointermediate lobe
NRS	Normal rabbit serum
OT	Oxytocin
PBS	Phosphate buffered saline
PNMT	Phenylethanolamine-N-methyl transferase
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
SCN	Suprachiasmatic nucleus
SS	Somatostatin
TRH	Thyrotrophin releasing hormone
VLGN	Ventral lateral geniculate nucleus
Bo	Total binding
MEA	0.2 M Mops[3-(N-morpholino) propane sulphonic acid] pH 7.0 50 mM sodium acetate 5 mM EDTA
MeOH	Methanol
NSB	Non-specific binding
TC	Total counts
TFA	Trifluoroacetic acid

CONTENTS

CHAPTER I

THE HYPOTHALAMIC-PITUITARY-ADRENOCORTICAL SYSTEM

Adrenocorticotrophin (ACTH) is secreted from the anterior pituitary and is under the control of a feedforward system which involves various components of the central nervous system. These components influence endocrine neurones in the hypothalamus which secrete corticotropin-releasing hormone (CRH), arginine vasopressin (AVP) and oxytocin into hypophysial portal blood. These neurohormones are transported to the anterior pituitary where they stimulate the release of ACTH and β-endorphin. ACTH stimulates the secretion of glucocorticoids from the adrenal cortex. The brain-hypothalamic-pituitary components of this system are modulated by the negative feedback of glucocorticoids. The neuroendocrine control of ACTH secretion will be discussed in this chapter.

1.1 Corticotrophin releasing factors

In 1948 Geoffrey Harris hypothesised that stress induced activation of the central nervous system is transduced from a neural into a humoral signal in the hypothalamus. Harris proposed that nervous stimuli may cause the liberation of a releasing factor into the capillary sinusoids of the median eminence, the releasing factor would then be transported via the hypophysial portal vessels to excite the anterior pituitary gland. More specific experimental evidence for the existence of a corticotrophin releasing factor (CRF) was obtained by Saffran & Schally (1955) and by Guillemin and Rosenberg (1955); however, their efforts to isolate and characterise the CRH were hampered by the lack of a CRF assay with sufficient accuracy and sensitivity and by the presence of vasopressin and catecholamines in hypothalamic extracts that had the ability to release ACTH. In 1970, Portanova & Sayers devised a

simple in vitro adenohypophysial cell dispersion technique for the measurement of CRF activity. An in vitro assay system was used by Vale et al. (1981) and culminated in the isolation and characterisation of a 41 amino acid peptide from ovine hypothalamus that was found to stimulate ACTH and β-endorphin secretion from primary cultures of anterior pituitary cells. Subsequently, the structures of rat (Rivier et al., 1983a) carp (Ling et al., 1984) and bovine CRH (Esch et al., 1984) have also been established and a structure proposed for human CRH on the basis of the sequence of the human CRH genome (Shibahara et al., 1983). Ovine and rat CRH differ by seven residues (Fig 1.1) while rat and human CRH are identical. Additionally, two other peptides show homologies with ovine CRH (Pallai et al., 1983); sauvagine, a 40-residue peptide found in frog skin and urotensin, a 41-residue peptide isolated from teleost urophyses. Comparative studies on the respective ability of these peptides to release ACTH have indicated that ovine CRH, urotensin and sauvagine exhibit equivalent potencies on rat corticotrophs (Rivier et al., 1983b).

The addition of increasing doses of ovine CRH to rat anterior pituitary cell cultures causes a dose-dependent increase in ACTH secretion (Rivier et al., 1983b). The action of CRH is mediated by cyclic AMP, and dexamethasone inhibits both the release of ACTH and the increase in pituitary cyclic AMP which are stimulated by CRH (Bilazikjian & Vale, 1983). Ovine CRH also stimulates secretion of other pro-opiomelanocortin (POMC) derived peptides from the anterior pituitary (Vale et al., 1983a). The presence of CRH in hypophysial portal plasma at concentrations sufficient to stimulate secretion of ACTH has been confirmed (Gibbs & Vale, 1982; Plotsky & Vale,

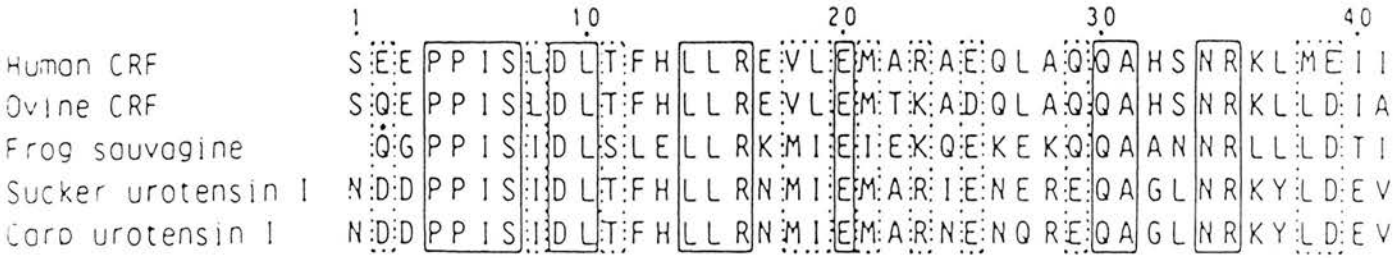


Figure 1.1 Comparison of amino acid sequences of human CRF, ovine CR, frog sauvagine, sucker urotensin I and carp urotensin I

Sets of identical residues are enclosed with solid lines and sets of residues considered to be favoured amino acid substitutions are enclosed with dotted lines. Favoured amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S; T.P.A. and G; N.D.E and Q; H,R and K; M.I.L. and V; F.Y. and W. The one-letter amino acid notation is used. Abbreviations are: S = serine; T = threonine; p = proline; A = alanine; G = glycine; N = asparagine; D = aspartic acid; E = glutamic acid; Q = glutamine; H = histadine; R = arginine; K = lysine; M = methionine; I = isoleucine; L = leucine; V = valine; F = phenylalanine; Y = tyrosine; W = tryptophan. After Shibahara et al. (1983).

1984). Exposure of rat anterior pituitary cells to ovine CRH for eight days results in an increase in ACTH content (cellular plus medium) of the cultures, suggesting CRH can elevate ACTH synthesis as well as release (Vale et al., 1983a). In addition, the finding that long-term treatment with ovine CRH increases POMC mRNA levels (Bruhn et al., 1984; Sutton et al., 1985) supports the hypothesis that CRH may regulate POMC gene expression.

1.1.1 Interaction between CRH and other ACTH secretagogues

The current concensus is that regulation of ACTH secretion is under multifactorial control. A variety of studies have indicated that the action of CRH on ACTH release can be modulated by glucocorticoids, catecholamines, neurohypophysial peptides and other substances. This hypothesis is supported by the observation that immunoneutralisation with antisera to ovine CRH did not abolish stress induced increases in ACTH secretion (Linton et al., 1983; Rivier & Vale, 1983a). The ability of AVP and, to a lesser extent, oxytocin (OT), which are themselves weak ACTH secretagogues, to potentiate the effect of CRH at the pituitary level has been observed many times (Antoni et al., 1983; Gillies et al., 1982; Vale et al., 1983). AVP and OT each have a synergistic action with CRH as shown by further elevation of ACTH secretion at plateau concentrations of CRH following the addition of AVP or OT. This synergistic effect is observed at concentrations of AVP and OT that are in the range of those found in portal blood (Horn et al., 1985). AVP and OT in portal blood are release from nerve terminals that project to the primary plexus of the portal vessels from neurones which, as is the case for CRH neurones, are located mainly

in the parvocellular region of the paraventricular nucleus (PVN) (Fig 1.2). The exact mechanisms involved in this potentiation are not fully understood. Although AVP does not appear to act on or by way of CRH receptors and does not by itself alter cyclic AMP accumulation in the anterior pituitary, it potentiates 4-fold the stimulation of cyclic AMP synthesis by CRH. This suggests that AVP may enhance the efficiency of coupling between the CRH receptor and adenylate cyclase (Giguere & Labrie, 1982a).

The catecholamines, adrenaline, noradrenaline and the β-adrenergic agonist isoprenaline have a smaller but still significant ACTH secretagogue activity relative to CRH (Vale et al., 1983; Rivier & Vale, 1985a). Simultaneous exposure of the pituitary gland to catecholamine and ovine CRH results in additive effects on ACTH secretion. In the case of adrenaline, this interaction takes place at concentrations of catecholamines reached in portal blood during stress (Gibbs, 1985) and may represent a physiological mechanism of ACTH secretion. Studies carried out with the aid of anti-CRH and anti-AVP sera and ganglionic blocking agents have shown that all three factors, CRH, AVP and catecholamines mediate stress-induced ACTH release, although of the three factors, CRH is the most potent (Rivier & Vale, 1983a; Linton et al., 1983). The interaction between catecholaminergic and CRH neurones is further complicated in that immunocytochemical studies have shown CRH immunoreactivity in the PVN increased after adrenaline synthesis had been blocked by a specific inhibitor of PNMT, suggesting that the adrenergic neurones that densely innervate CRH neurones in the PVN inhibit CRH release (Mezey et al., 1984).

Angiotensin II has also been shown to be a weak ACTH

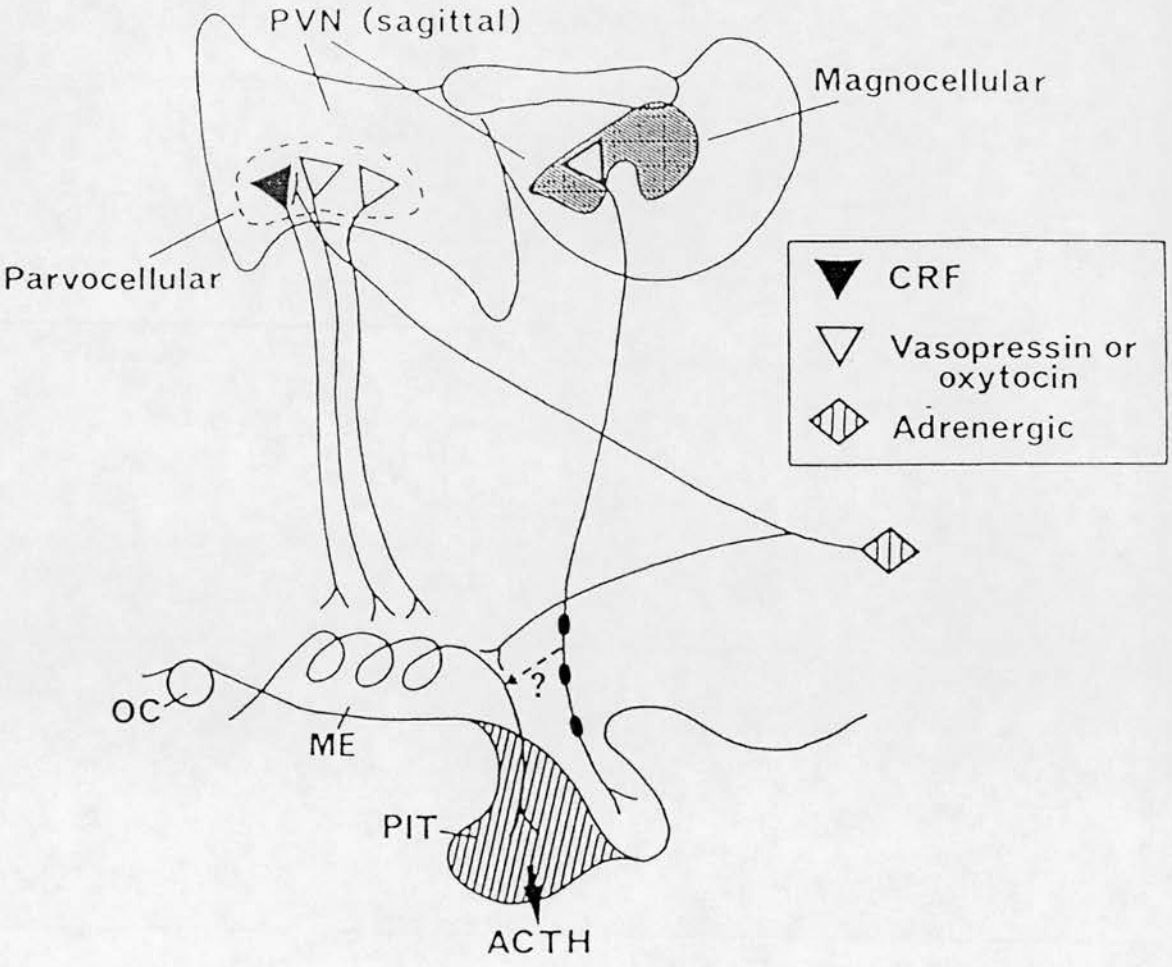


Figure 1.2 Schematic diagram of a sagittal section of the hypothalamus

Sagittal section of the hypothalamus illustrating the major neurones involved in the control of ACTH release. Ascending adrenergic projections may inhibit CRF release by actions on the cell body, but probably release ACTH by a direct action on anterior pituitary cells. For clarity, other modulating neurones (GABAergic, opioid and noradrenergic) are not shown nor are the controlling inputs from the limbic or other areas of the CNS. Vasopressin and oxytocin, as well as being released from terminals on the portal vessels, may also be released laterally from fibres in the supraoptic-hypophysial tract. (PVN = paraventricular nucleus; ME = median eminence, with the primary plexus of the hypophysial portal vessels; Pit = pituitary gland).

secretagogue (Rivier & Vale, 1983b; Spinedi & Negro-Vilar, 1984) and also enhances slightly the ACTH response to CRH (Rivier & Vale, 1983b). Angiotensin II immunoreactivity is found in PVN neurones that project to the external layer of the median eminence and the median eminence content of angiotensin II increases after adrenalectomy. However, pharmacological blockade of receptors to angiotensin II did not affect basal or stress induced concentrations of ACTH and corticosterone (Buckner et al., 1986) and there have been no reports of angiotensin II release into hypophysial portal blood.

Somatostatin (SS) is present in hypophysial portal blood in significant concentrations (Sheward et al., 1984) and, therefore, a possible interaction between SS and CRH on ACTH secretion has been investigated. Richardson (1983) has observed that SS significantly inhibits CRH induced ACTH release from mouse pituitary tumour cells, however, the doses necessary to achieve this inhibition are far higher than reported to be present in portal blood.

A variety of in vitro studies indicate that the stimulatory effect of CRH on the corticotrophs is inhibited by glucocortoicds via feedback inhibition, a process classically considered to be both time- and concentration-dependent (Keller-Wood & Dallman, 1984). This effect appears to be near maximum if cells are incubated with dexamethasone for 4h, but only partial if dexamethasone is added at the same time as ovine CRH. Increasing doses of steroids shift the dose-response curves to ovine CRH to the right and reduce the maximum stimulated ACTH release (Vale et al., 1983a). In vitro, CRH can still stimulate some ACTH secretion even at maximally effective concentrations of glucocorticoids. This correlates with

studies in vivo showing that stress (which presumably increases CRH secretion) is capable of increasing plasma ACTH concentrations even in the presence of high circulating corticosterone concentrations (Plotsky & Vale, 1984).

1.1.2 Control of CRH release

1.1.2.1 Neurotransmitter effects

Understanding the central neurotransmitters involved in controlling CRH release has been complicated by the multiple hypothalamic factors which regulate ACTH secretion and by the necessity to use bioassays to determine CRH activity which often did not discriminate between these multiple factors. However, development of a sensitive radioimmunoassay for CRH has reduced these problems. A general summary of the data accumulated suggests that CRH release is facilitated by central cholinergic and serotonergic neurones and is inhibited by adrenaline, GABA, morphine and β-endorphin (Rivier & Plotsky, 1986). The role of noradrenaline is more controversial in that noradrenaline has been generally thought to inhibit the secretion of bioactive CRH (Berkenbosch et al., 1981; Weiner & Ganong, 1978;) but studies in the rat and in man suggest that noradrenaline facilitates CRH release (Hary et al., 1984; Rivier & Plotsky, 1986).

More direct evidence on the effect of neurotransmitters has been provided by measurements of CRH release into portal blood. Information gained from studies on neurotransmitter release into portal blood is not free from artefact, as apart from anaesthesia and surgical stress, pharmacological agents injected systemically

or into cerebral ventricles could exert indirect effects on neurotransmitter release. With this in mind, the facilitatory role of the serotonergic system has been demonstrated by Gibbs & Vale (1983) who reported that blockade of re-uptake of serotonin into presynaptic terminals with fluoxetine significantly increased the release of AVP and CRH into portal blood. Plotsky has reported preliminary results that indicate acetylcholine is associated with a 3-fold elevation of CRH release into portal blood while noradrenaline caused a decrease in portal blood CRH concentrations. Administration of β-endorphin significantly reduced portal CRH concentrations while intracerebroventricular injection of naloxone increased 2.5-fold the CRH concentration in portal blood, suggesting that opiates may act as autoregulatory agents to limit CRH secretion (Rivier & Plotsky, 1986). Vasopressin administration has been found to decrease secretion of CRH into portal blood in a dose-dependent fashion (Plotsky et al., 1985), suggesting that endogenous AVP has an inhibitory influence at the central level while acting at the pituitary level to potentiate the ACTH releasing activity of CRH.

1.1.2.2 Differential release and action of CRF, AVP and OT

ACTH secretion is controlled by multiple factors and flexibility of the neural regulation of ACTH secretion has been studied by the direct measurement of the various releasing factors into portal blood in response to various stimuli. Haemorrhage is associated with increased portal blood concentrations of CRH, AVP, OT and adrenaline (Poltsky et al., 1985b; Plotsky & Vale, 1984). The ACTH secretory response to haemorrhage is attenuated by

pre-treatment with either CRH antiserum, AVP antagonists or ganglionic blockers (Plotsky, 1985a; Plotsky et al., 1985b), suggesting that the regulation of haemorrhage-induced secretion is multifactorial in nature and that CRH acts as the predominant regulatory agent. Insulin-induced hypoglycemia is frequently used to test the integrity of the hypothalamic-adrenocortical system. In the fasted rat, the ACTH response to insulin-induced hypoglycaemia is abolished by anti-CRH serum, attenuated by administration of a AVP antagonist and moderately reduced by ganglionic blockade (Plotsky et al., 1985c). However, insulin-induced hypoglycemia is accompanied by a significant increase in AVP but not CRH or OT concentrations in portal blood (Plotsky et al., 1985c) suggesting that in this situation, CRH plays a permissive role which is necessary for an effective ACTH response to the less potent ACTH secretagogue, AVP.

The apparent differential effects of CRH and AVP are made more complex by the recent findings that 44% of immunoreactive CRH terminals in the external layer of the median eminence of the rat also stain strongly for AVP and AVP-neurophysin and that after adrenalectomy virtually all CRH positive terminals were also positive for AVP and AVP-neurophysin. Co-localisation of CRH, AVP and AVP-neurophysin in many neurosecretory vesicles in nerve terminals of the median eminence has also been demonstrated by electron microscopy (Whitnall et al., 1985).

1.1.2.3 CRH receptors

Specific, high-affinity receptors for CRH have been localised in the anterior and intermediate lobes of the rat and human pituitary

gland (de Souza et al., 1985a). CRH receptors in the anterior pituitary parallel the distribution of corticotrophs in the anterior pituitary, whereas receptors in the intermediate lobe are distributed homogeneously. The CRH receptors appear to be coupled to the adenylate cyclase system (Giguere et al., 1982b; Labrie et al., 1983). After adrenalectomy there is a decrease in the CRH receptor concentration in the rat anterior pituitary gland which is associated with a comparable decrease in maximal CRH-stimulated adenylate cyclase activity and sensitivity to CRH. These decreases in receptor density and adenylate cyclase activity are presumably due to down-regulation of the CRH receptor and can be prevented by treatment with dexamethasone (Wynn et al., 1985). CRH receptors are also present in the cerebral cortex and parts of the limbic system in rat brain and these receptors appear to be unaffected by adrenalectomy (de Souza et al., 1985b).

Specific CRH receptors have also been found in monkey adrenal medulla and sympathetic ganglia. In the adrenal medulla, these receptors are coupled to adenylate cyclase and stimulate the secretion of catecholamines and met-enkephalin. This supports the view that CRH has a paracrine action in peripheral tissues (Udelsman et al., 1986).

1.1.3 Extra pituitary actions of CRH

Intracerebroventricular administration of ovine CRH *in vivo* has provided evidence that CRH may have an extra pituitary site of action. Mean arterial pressure and heart rate increased after intraventricular administration of ovine CRH (Fisher et al., 1982) as did plasma concentrations of adrenaline, noradrenaline, glucagon

and glucose (Brown et al., 1982). Behavioural activation was also observed (Britton et al., 1982; Sutton et al., 1982).

In vivo, CRH has also been shown to influence the secretion of other pituitary hormones by interactions with various transmitters at the hypothalamic level. Intracerebral injection of ovine CRH on the morning of pro-oestrus attenuates the LH and GnRH surge (Rivier & Plotsky, 1986) suggesting CRH may have an inhibitory effect on central release of GnRH. In the rat, intracerebral injection of ovine CRH decreased plasma growth hormone concentrations and this was reversed by immunoneutralisation with anti-SS serum (Katakami et al., 1985; Rivier & Vale, 1985b).

The character of pituitary and extra pituitary actions of CRH is consistent with the concept of CRH as a peptide primarily concerned with the physiological changes that help the animal respond to stress and may explain the deleterious action of stress on reproductive functions.

1.2 Adrenocorticotrophin and related peptides

1.2.1 Biosynthesis

The primary structure of ACTH was first determined in 1954 and found to be a peptide hormone comprising 39 amino acids with a molecular weight of 4.5K (Bell, 1954). Pulse chase studies demonstrate that ACTH is synthesised as part of a larger precursor with a Mr of 31K which is processed to 4.5K Mr ACTH through intermediate forms (Mains & Eipper, 1976). Translation of pituitary mRNA in a cell-free system followed by immunoprecipitation yielded the initial precursor of ACTH with Mr of 35K (Nakanishi et

al., 1976). Further cell-free translation studies showed that the ACTH precursor encoded by pituitary mRNA also contained β-endorphin (Nakanishi et al., 1977a). β-endorphin had previously been shown to be derived from β-lipotropin (β-LPH) (Li & Chung, 1976) and their data showed that there is thus a common precursor for both ACTH and β-LPH. The structure of the ACTH/β-LPH precursor pro-opiomelanocortin (POMC), was finally determined from the nucleotide sequence of cloned DNA complementary to the mRNA coding for the precursor (Nakanishi et al., 1979) (Fig. 1.3). The precursor is enzymatically processed to give ACTH, which can be further processed to αMSH and corticotropin-like intermediate peptide (CLIP) and β-LPH which can be further processed to produce β-endorphin and γ-LPH. β-endorphin could theoretically be further processed to methionine-enkephalin but there is no evidence to support the view that POMC is a source of enkephalin. POMC is glycosylated and its products can be further modified by glycosylation, phosphorylation, sulphation, N-terminal acetylation and C-terminal cleavage (Liotta & Krieger, 1983; Hollt, 1985b; Jenks et al., 1986).

Post-translational patterns of processing POMC are tissue specific. In the anterior pituitary, POMC is cleaved to form mainly ACTH, β-LPH and N-terminal peptide, β-LPH is processed to a limited extent to form γ-LPH and β-endorphin (Fig. 1.4). In the intermediate lobe of the pituitary, ACTH is processed to form α-MSH and CLIP, N-terminal peptide is converted to some extent to γ-MSH and the remaining amino terminal portion. The conversion of β-LPH to γ-LPH and β-endorphin is almost complete and further processing may occur to yield biologically inactive forms of β-endorphin and

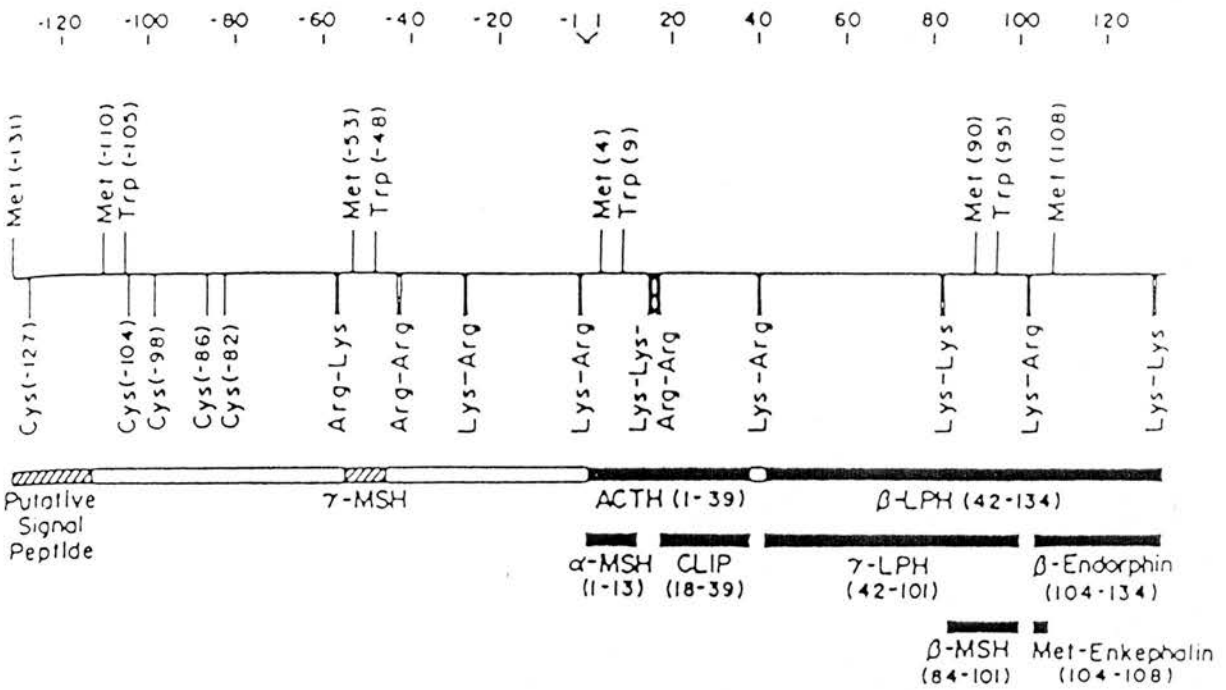


Figure 1.3 Schematic representation of bovine ACTH-β-LPH precursor

Characteristic amino acid residues are shown and the positions of the methionine, tryptophan and cysteine residues are given in parenthesis. The closed bars represent the regions for which the amino acid sequence were known and the open and shaded bars represent the regions for which the amino acid sequence has been predicted from the nucleotide sequence of the ACTH-β-LPH precursor mRNA. The locations of known peptides are shown by closed bars and the amino acid numbers given in parenthesis. From Nakinishi et al. (1979).

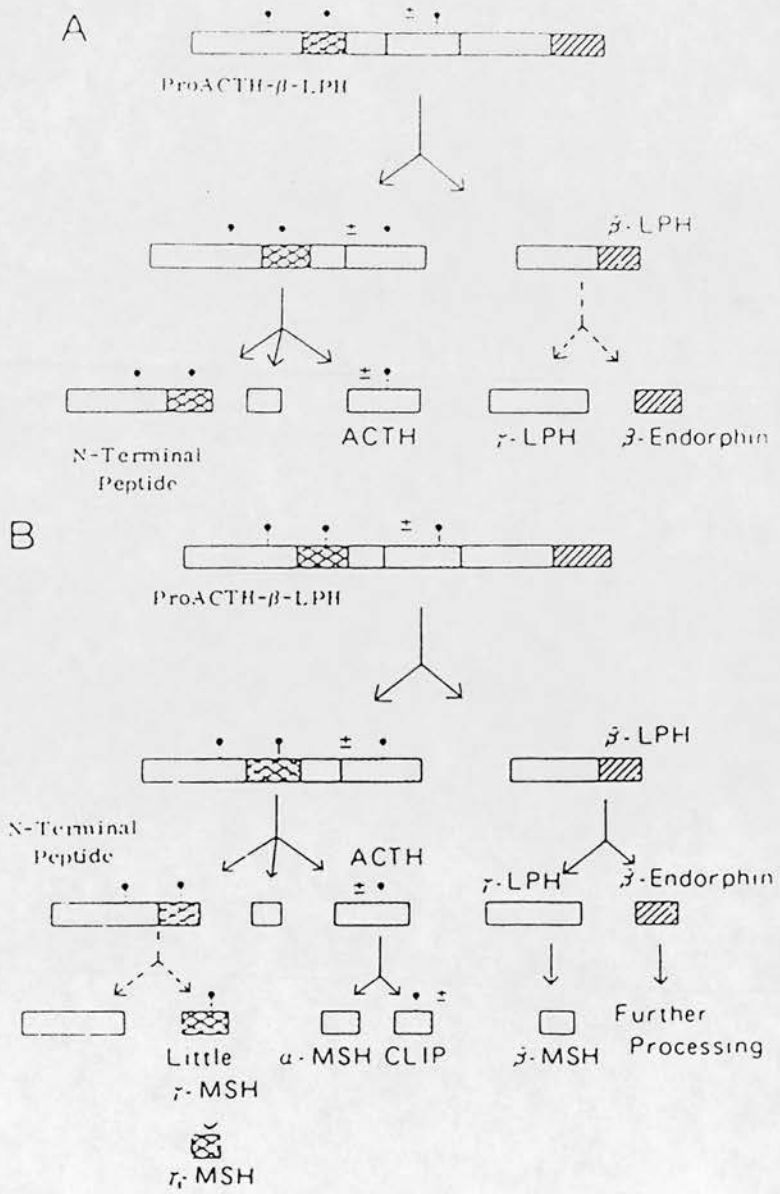


Fig. 1.4 Schematic representation of tissue specific processing of the ACTH-β-LPH precursor

Processing of pro-ACTH-β-LPH in the anterior pituitary (A) and in the neurointermediate pituitary and extrapituitary tissues (B). Dotted lines represent limited processing. From Imura et al., (1983).

β-MSH from γ-LPH (Imura et al., 1983).

There is widespread distribution of POMC fibres throughout the brain, most of which are presumed to derive from cell bodies in the arcuate nucleus. Hypothalamic nuclei, in particular the PVN, receive an especially dense innervation of POMC-containing fibres as does the median eminence. Several of the hind and mid brain nuclei and the periaqueductal grey are also densely innervated with POMC containing fibres (Liotta & Krieger, 1983). The processing patterns of POMC in the brain have been shown to be similar to those in the intermediate lobe of the pituitary gland (Imura et al., 1982). The physiological significance of POMC yielding multiple bioactive hormones may be that they perform co-ordinated modulatory functions in the central nervous system and peripheral tissues in response to stress.

In summary, ACTH and β-endorphin are derivatives of a 31K glycosylated precursor protein, POMC which undergoes post-translational processing. Post translational processing and modification is tissue specific and serve as important regulatory mechanisms. Acetylation, which is catalysed by acetyltransferase, is an important regulatory mechanism. For example, in the intermediate lobe of the frog, dopamine inhibits acetylation resulting in the presence of inactive (des-acetyl-α-MSH) and active β-endorphin. In the uninhibited state all the α-MSH is acetylated and, therefore, active. Since N-acetylated β-endorphin is inactive, this mechanism could operate to provide a switch from a potent melantrophic signal to an opioid signal (Jenks et al., 1986).

1.2.2 Regulation of POMC synthesis

Recent evidence indicates that factors known to alter secretion of POMC peptide hormones may also alter POMC biosynthesis via modulation of POMC mRNA levels. Differential regulation of POMC release and synthesis occurs in the anterior and intermediate lobes of the pituitary gland. Removal of glucocorticoids by adrenalectomy increased POMC mRNA significantly in the anterior pituitary gland and POMC mRNA was reduced by treatment with dexamethasone (Herbert et al., 1981). Glucocorticoids have been shown to exercise their action at the level of transcription, changes in transcription rates of POMC were visible 10-15 min after adrenalectomy (Eberwine & Roberts, 1984). Long-term treatment with CRH results in increased POMC mRNA content in the anterior pituitary gland (Bruhn et al., 1984). Glucocorticoids or CRH have little or no effect on POMC mRNA content in the intermediate lobe.

POMC gene expression in the intermediate lobe can be altered by dopamine, blockade of the dopamine receptor by haloperidol increased, while a dopamine agonist, ergocryptine, decreased the content of POMC mRNA in the intermediate lobe of the pituitary gland (Chen et al., 1983). The mechanism of action is unclear but may be mediated by cyclic AMP.

POMC gene expression is also affected by other factors such as oestrogen which decreases both content of POMC mRNA and rate of POMC transcription in the medial basal hypothalamus in ovariectomised rats. Presumably the main site of action of oestrogen is the arcuate nucleus as that is where, in the hypothalamus, the cell bodies of POMC-containing neurones are concentrated (Roberts et al., 1986).

1.2.3 Function and mechanism of action of POMC derived peptides

ACTH, released from the anterior pituitary gland, has its main endocrine action on the adrenal gland where it stimulates the secretion and synthesis of glucocorticoids and to a lesser extent mineralo corticoids. ACTH also stimulates cell growth and division in the zona fasciculata and reticularis. These actions are produced by ACTH binding to receptors in the plasmalemma which results in the production of cyclic AMP (Sayers et al., 1974) which, in turn, activates cAMP-dependant protein kinases. Activated kinase may phosphorylate a cholesterol esterase which accelerates the conversion of cholesterol fatty acid esters in lipid droplets of adrenocorticoid cells to free cholesterol. Cholesterol in the cellular part of free cholesterol may also be derived from plasma. Cyclic AMP is believed to induce the synthesis of a labile protein which accelerates the conversion of cholesterol to pregnenolene (the rate limiting step in glucocorticoid synthesis) possibly by transporting cholesterol to its site of chain cleavage on the mitochondrial P450 system. Once pregnenolene is formed, the subsequent steroid conversions follow at a rapid rate. Activation of protein kinase also promotes cell division. In addition to cyclic AMP, Ca^{2+} is involved in the mechanism of action of ACTH.

The physiological functions of other POMC derivatives are not clear. MSH stimulates melanocytes in lower vertebrates and possibly under pathological conditions in man, γ-MSH has been reported to potentiate the adrenal response to ACTH (Al-Dujaili et al., 1981) and β-MSH has been reported to stimulate aldosterone but not corticosterone production (Matsuoko et al., 1981). The role, if any, of β-endorphin in acupuncture has been widely discussed, but

is yet to be proven (Clement-Jones, 1982) and studies on the role of β-endorphin in affective disorders and schizophrenia are inconclusive (Berger et al., 1986). Studies in genetically obese mice suggest that CLIP-like peptide may stimulate insulin secretion (Beloff-Chain et al., 1983). Finally, the placenta synthesises large amounts of POMC but its role remains uncertain (Krieger et al., 1980).

1.3 Glucocorticoids

1.3.1 Secretion and function of glucocorticoids

In man, dogs and guinea pigs, the main glucocorticoid secreted is cortisol while in the rat, mouse and cat, corticosterone is the predominant glucocorticoid. Glucocorticoids are secreted from the zona fasciculata and reticularis of the adrenal cortex and have very diverse functions. They induce enzymes such as tryptophan oxidase, phenylethanolamine N-methyl transferase, glutamine synthetase and hepatic cytochrome P-450. Glucocorticoids raise blood glucose by stimulating hepatic gluconeogenesis, inhibiting glucose uptake in peripheral tissues, suppressing insulin and stimulating glucagon, all of which result in increased blood glucose. The increase in blood glucose is prolonged by the synergistic action of glucocorticoids with glucagon and adrenaline. Glucocorticoids also suppress inflammation, the immune response and the activity of plasminogen activator and other neutral proteinases (Munck et al., 1984). Glucocorticoids also inhibit the secretion of ACTH and other derivatives of POMC as part of negative feedback control (Herbert et al., 1981).

1.3.2 Functions of glucocorticoids in stress

Almost any kind of threat to homeostasis or stress will cause plasma glucocorticoid concentrations to rise. The increased concentrations have traditionally been ascribed the physiological function of enhancing the organism's resistance to stress, a role well recognised in glucocorticoid therapy. How the known physiological and pharmacological effects of glucocorticoids might accomplish this function has been reviewed by Munck et al. (1984).

Contrary to the traditional view that glucocorticoids enhance defence mechanisms, it has become increasingly clear that glucocorticoids at moderate to high concentrations generally suppress the defence mechanisms. The potent antiinflammatory action of glucocorticoids, which depends on the ability of these steroids to suppress the various cellular and humoral defence systems of the body, would appear to conflict with the role of glucocorticoids in protecting the organism from stress. Munck et al. (1984) argue that stress-induced increases in glucocorticoid concentrations protect not against the source of stress itself but rather against the body's normal reactions to stress, preventing these reactions from overshooting and threatening homeostasis. They support this hypothesis by arguing, for example, that glucocorticoids can suppress mediators of inflammation and immune reactions such as immune interferon, lymphocyte activating factor, T cell growth factor, prostaglandins, leukotrienes, histamine, 5-HT, bradykinin and neutral proteinases. The glucocorticoid response is delayed relative to the onset of an immune response, this allows the immune response to be mounted and then subsequently moderated by glucocorticoids. In the case of a metabolic challenge, Munck et

al. (1984) argue that the major metabolic role of glucocorticoids is to prevent insulin from causing dangerous hypoglycaemia. Glucocorticoids act synergistically with glucagon and adrenaline in that the glucocorticoids (which act more slowly) enhance and prolong the increase in blood glucose initiated by adrenaline and glucagon. The glucocorticoids raise blood glucose by stimulating hepatic gluconeogenesis, inhibiting glucose uptake by peripheral tissues, inhibiting the secretion of insulin and stimulating the secretion of glucagon.

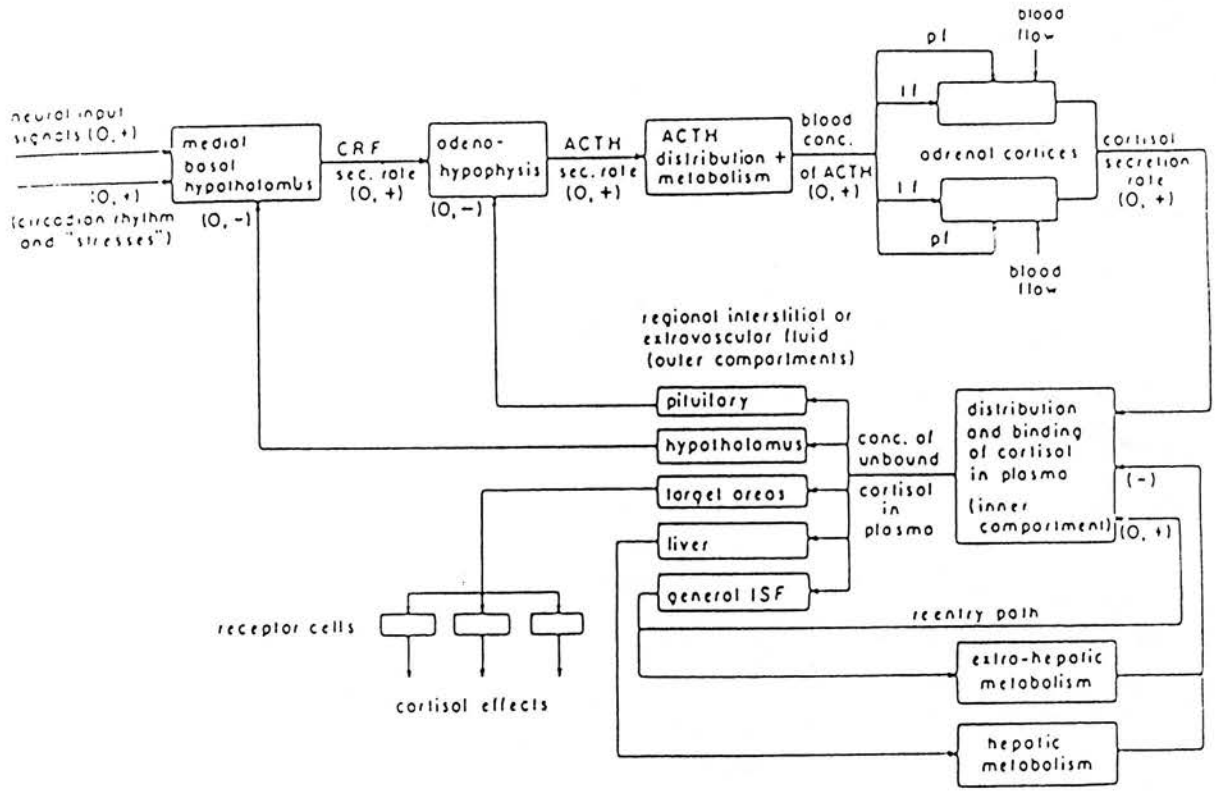
This hypothesis provides glucocorticoid physiology with a unified conceptual framework that can accommodate disparate glucocorticoid effects on carbohydrate metabolism, immune reactions, water balance, shock and levels of enzymes, though care must be taken to avoid erroneous generalisations.

1.3.3 Control of ACTH secretion by glucocorticoids

The secretion of ACTH is under negative feedback control of glucocorticoids. The existence of this negative feedback, homeostatic system was established in the 1930's and 1940's (Sayers, 1950) and developed into a sophisticated model by Yates and colleagues (Yates & Brennan, 1967; Yates et al., 1969) which is illustrated in Fig. 1.5. The amount of ACTH which reaches and acts on the adrenal cortex depends on the rate of its secretion, distribution in body compartments and metabolic clearance. This is further complicated in glucocorticoids as it is generally assumed that only free glucocorticoid is active in the negative feedback control of the brain-pituitary component. In man, only 8% of glucocorticoids are not bound to plasma proteins (Bondy, 1985).

Figure 1.5 Model of the adrenal glucocorticoid control system as devised by Yates et al., 1969.

Studies on the release of CRF and ACTH suggest that rapid glucocorticoid inhibition of the system occurs at the level of the anterior pituitary gland: long term effects may also occur at the medial basal hypothalamus and possibly other areas of the brain. Yates et al. used the abbreviations 'if' for 'input forcing' of the adrenal by ACTH and 'pf' for 'parametric forcing' of the adrenal (hypertrophic effect). The latter is too slow for it to play a significant role in the ACTH-glucocorticoid response to stress.


neural input signals (0, +)
(circadian rhythm and "stresses")
medial basal hypothalamus
(0, −)
CRF sec. rate (0, +)
adeno-hypophysis
(0, −)
ACTH sec. rate (0, +)
ACTH distribution + metabolism
blood conc. of ACTH (0, +)
pl
blood flow
adrenal cortices
cortisol secretion rate (0, +)
regional interstitial or extravascular fluid (outer compartments)
pituitary
hypothalamus
target areas
liver
general ISF
conc. of unbound cortisol in plasma
distribution and binding of cortisol in plasma (inner compartment)
(−)
(0, +)
reentry path
receptor cells
cortisol effects
extra-hepatic metabolism
hepatic metabolism

The glucocorticoid binding proteins serve as a buffer and ensure the transport of the relatively hydrophobic glucocorticoids to specific tissue sites; glucocorticoid binding globulin, presumably with bound steroid, has been shown to enter liver and pituitary cells (de Kloet & Reul, 1986). The marked changes in plasma cortisol during circadian rhythm shows there is rapid and free exchange of bound and free glucocorticoid.

Corticosteroid feedback inhibition of stress-induced ACTH secretion has been shown to operate in at least three time domains: fast feedback (within seconds to minutes), intermediate (over 2-10h) and slow (over hours to days). Fast feedback was first postulated to be rate-sensitive by Dallman & Yates (1969) and was confirmed experimentally by Jones et al. (1972) and by Abe & Critchlow (1977). Both groups found that rapid inhibition of stress-induced ACTH secretion occurred during the period of increasing plasma corticosteroid concentrations and that the inhibition had disappeared by 30 min after injection. There then followed a 'silent period' during which no inhibition of adrenocortical response to stress is observed (Dallman & Yates, 1969). Intermediate feedback occurs 90-120 min after the rise in corticosteroids. Takebe et al. (1971) and Jones et al. (1974) have shown the degree and duration of intermediate inhibition depends on the total dose of steroid administered and that maximal inhibition of adrenocortical responses occur between 2h and 4h after the administration of a single dose of corticosterone. Intermediate feedback is followed by slow feedback, sustained elevation of corticosteroids for one or more days abolishes the capacity of the adrenocortical system to respond to most stimuli (Buckingham &

Hodges, 1974). Slow feedback, however, would be expected to occur only in pathological conditions or after pharmacological treatment with corticosteroids when increases in plasma corticosteroids are prolonged for days.

Studies on basal release of ACTH suggest basal ACTH release is less sensitive to corticosteroid feedback inhibition. In studies designed to examine rapid inhibition of ACTH by glucocorticoids in vitro no inhibition was observed unless the pituitary was stimulated (Widmaier & Dallman, 1983). Inhibition of basal ACTH release in vivo by glucocorticoids has been demonstrated during the 'silent period' (Zimmerman & Critchlow, 1972). Basal ACTH release is suppressed 20 min after administration of glucocorticoids in dogs and after 45 min in man. It appears, therefore, that basal ACTH secretion in normal animals is inhibited by corticosteroids and that the temporal pattern for inhibition of this secretion is different in rats and man than that for inhibition of stimulus-induced secretion.

1.4 Feedback control of the ACTH-glucocorticoid circadian rhythm

The circadian rhythm in basal secretion of glucocorticoids and ACTH is a predominant characteristic of the adrenocortical system. With the exception of dogs, circadian rhythms in plasma ACTH have been reported in all species studied (Gallagher et al., 1973; Krieger et al., 1971; Rees et al., 1971). Peak activity of the system occurs at the onset of the daily activity cycle, for example, in man the peak is between 0500-0800h and in nocturnal animals such as the rat, the rhythm is reversed with the peak occurring towards the end of the light phase (Chiappa & Fink, 1977). There is

evidence that at the time of the daily adrenocortical minimum, pituitary ACTH secretion does not require input from the brain. Lesions placed in the medial basal hypothalamus of the rat, which prevented stimulus-induced ACTH secretion, had no effect on normal resting ACTH levels in the morning, but prevented the increase in ACTH secretion normally seen towards the end of the light phase (Kaneko et al., 1980). Studies in experimental animals and man show that the rhythm is driven by a central neural mechanism of which the main components are the suprachiasmatic nuclei (SCN) (Moore, 1979; Krieger, 1979a). That is, the SCN generate a rhythm which approximates to 24h and this rhythm is transduced into a circadian rhythm of the release of CRF's which, in turn, results in a 24h rhythm in the release of ACTH and consequently glucocorticoids.

There are several factors which entrain the hypothalamic-pituitary-adrenocortical rhythm, of which light is the most prominent (Moore, 1979; Krieger, 1979a), but it remains to be determined precisely how the circadian rhythm is generated and how signals are transmitted from the SCN to CRF neurones. The SCN receive prominent projections from the midbrain raphe nuclei in which 5-HT is the neurotransmitter. The SCN also has prominent reciprocal connections with the ventral division of the lateral geniculate nuclei (VLGN). The functions of the connections between the SCN and the raphe nuclei and VLGN are not known, but the SCN, VLGN and hippocampus, all of which have a relatively dense 5-HT innervation, show circadian changes in the electrophysiological response to 5-HT (Mason, 1986). Lesions of the raphe nuclei do not alter the frequency but reduce by about 50% the amplitude of the ACTH circadian rhythm (Szatarczyk et al., 1980). This suggests

that the raphe nuclei alter the magnitude but not the frequency of the signal generated by the SCN.

The technical problems of measuring CRH in hypophysial portal blood make it difficult to prove that the circadian rhythm of ACTH and corticosterone is in fact driven by brain derived CRH However, changes in CRF activity (as determined by bioassay) of hypothalamic extracts are consistent with a neural drive for the circadian rhythm which is mediated by way of CRF's (Chiappa & Fink, 1977).

1.4.1 Receptors for corticosteroids

Most of the actions of glucocorticoids are thought to be genomic and depend on the binding on the steroid with a cytosolic receptor and the movement of the steroid receptor complex to the nucleus where it binds to chromatin and either promotes or suppresses gene expression (Miesfeld et al., 1986). There are two types of glucocorticoid receptors in the rat (de Kloet & Reul, 1986). Type 1 (first described by McEwan et al., 1968) bind ^{3}H-corticosterone and ^{3}H-aldosterone and are concentrated in CA1 and CA2 regions of the hippocampus and other areas of the limbic system. There are only a few type 1 receptors in the hypothalamus or pituitary gland and this is surprising given the presumption that feedback of glucocorticoids is exerted at the level of the hypothalamic-pituitary systems. However, type 2 receptors bind ^{3}H-dexamethasone and ^{3}H-RU28362 (a synthetic ligand for glucocorticoid receptors), are similar to the liver glucocorticoid receptor as identified by monoclonal antibodies (Fuxe et al., 1985) and are widely distributed in neurones and glial cells all over the telecephalon, diencephalon and anterior pituitary gland.

Concentration of these receptors, as assessed by immunocytochemistry, is not high, except in the PVN, the anterior periventricular nuclei, the ventral mediobasal hypothalamus and CA1 and CA2 region of the hippocampus. Overlap of glucocorticoid receptor density and immunoreactivity in hypothalamic areas has been proposed as evidence that glucocorticoids play a role in the secretion of CRH, TRH and somatostatin (Fuxe et al., 1985).

The differentiation of receptor systems for glucocorticoids suggest a dual role of the naturally occurring hormone, corticosterone, in the control of brain function. Type 2 receptors have been shown to become occupied with rising plasma concentrations of corticosterone after stress. This implies that Type 2 receptors mediate the feedback action of glucocorticoids aimed to turn off stress-activated brain mechanisms. The negative feedback role is supported by high concentrations of Type 2 receptors in the anterior pituitary gland and in the hypothalamus. Type 1 receptors are concentrated mainly in the limbic system and corticosterone action on Type 1 receptors may be associated with cognitive functions ascribed to the limbic system. Differential function of the two receptor types requires proof that both receptors recognise the naturally occurring corticosterone equally.

1.5 Aims of this Thesis

There were two main aims of this thesis. First, I wished to examine, with the aid of the technique of hypophysial portal blood collection, the interplay between and the relative importance of the various CRF's in stimulating ACTH *in vivo*. Specifically, direct measurements of CRH, AVP and OT release into hypophysial portal

blood were made to examine (i) the role of CRH and AVP in mediating ACTH secretion in response to electrical stimulation of the amygdala, hippocampus, median eminence and PVN, (ii) the site of action of glucocorticoids in mediating their negative feedback action, (iii) the role of endogenous opioid peptides in inhibiting release of AVP and OT into portal blood during electrical stimulation of the median eminence and iv) the effect of the presumed absence of AVP in the Brattleboro rat on CRH and OT release into portal blood and on ACTH release from the anterior pituitary gland. Secondly, I wished to investigate the factors that affect the synthesis of POMC and related peptides by measuring the content of POMC mRNA in the anterior pituitary gland in response to low and high concentrations of corticosterone, in response to electrical stimulation of CRH release into hypophysial portal blood and in the Brattleboro rat which is genetically deficient in AVP.

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 ANIMALS

Animals used in this study were adult female Wistar cobs (caesarean-originated barrier-sustained) and adult female Long-Evans rats purchased from Charles River UK Ltd. (Margate, Kent). Female homozygous and heterozygous Brattleboro rats were bred in the Department of Pharmacology, Edinburgh. Animals were housed in cages of four under controlled lighting (lights on 05.00-19.00h) and temperature (22°C) and had free access to food (Diet 41B; Oxoid, Basingstoke) and tap water. All animals subjected to surgery and maintained alive for more than 48h had access to drinking water supplemented with aureomycin (50mg/litre) (Chlortetracycline hydrochloride; Cynamid G.B. Ltd., Gosport). Long-term adrenalectomised animals received 0.9% saline supplemented with glucose (250mg/litre) and aureomycin instead of tap water.

Brattleboro rats were caged singly in metabolic cages to measure urine output in a 24h period. A homozygous Brattleboro rat was defined as an animal which passed urine equal to or greater than 75% of body weight during the 24h collection period. Animals passing less than this were classed as heterozygous Brattleboro rats.

2.2 SURGICAL AND EXPERIMENTAL PROCEDURES

2.2.1 Anaesthetics

The following anaesthetics were used:

1) Ethyl carbamate (urethane; Sigma Chemical Co. Ltd., Poole, Dorset). A 10% (w/v) solution was prepared in 0.9% saline and administered by intraperitoneal (i.p.) injection at a dose of 1.0-1.2g/kg body weight.

2) Sodium pentobarbitone (sagatal; May & Baker Ltd., Dagenham). Purchased as 60mg/ml sodium pentobarbitone in alcohol: propylene glycol vehicle (10:20 v/v), diluted 1:10 in 0.9% saline and injected i.p. at a dose of 36-40mg/kg body weight.

3) 9 mg/ml alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione and 3 mg/ml pregnane-11, 20-dione) solubilised in saline by 20% (w/v) polyoxyethylated castor oil (saffan; Glaxovet Ltd., Uxbridge). Administered i.p. at a dose of 1ml/100g body weight.

4) 2,2,2-tribromoethanol (Aldrich Chemical Co. Gillingham, Kent) in amylene hydrate (tetr. amyl alcohol; BDH, Poole, Dorset) 1g/ml; 2ml of the stock solution were diluted with 8ml absolute alcohol and 90ml 0.9% saline solution. The dose used was 1.0ml/100g body weight administered by i.p. injection.

5) Anaesthetic ether (McFarlane Smith Ltd., Edinburgh).

2.2.2 Drugs

1. Dexamethasone (Sigma Chemical Co. Ltd.). A stock solution of 0.9mg/ml in ethanol was diluted 1:10 (v/v) with 0.9% saline solution immediately before use and injected i.p. at a dose of 0.5mg/kg body weight.
2. [1-deamino-8-D-arginine]-vasopressin: dDAVP (Ferring, Malmo, Sweden). Stock solution of 10μg/ml injected i.p. at a dose of 10μg/100g body weight.

2.2.3 Hypothalamus and Pituitary Dissection

Rats were decapitated and brains quickly removed and placed on ice ventral side uppermost. The optic nerves were removed at the

level of the optic chiasm with fine forceps before the hypothalamus was dissected out. A block of tissue about 2-3mm deep was removed extending rostro-caudally from the pre-optic area to just caudal to the mamillary bodies and laterally 1mm on either side of the median eminence. The block of tissue generally weighed between 28 and 35mg.

The pituitary gland was separated into anterior lobes and neurointermediate lobes in situ with the aid of watchmaker forceps and under a dissecting microscope.

2.2.4 Adrenalectomy

Female Wistar cobs (200-250g body weight) were adrenalectomised in two stages. The second adrenal gland was removed 7-10 days after the first gland. The survival rate using this approach was higher than immediate bilateral adrenalectomy.

On day 1, animals were anaesthetised with tribromoethanol and an incision made along the right costal margin. The right kidney was exposed and the adrenal gland excised. Bleeding was stopped by gentle pressure with a gauze swab and the incision was sutured. On day 7-10, animals were anaesthetised as before and the left adrenal gland removed in the same manner as the right gland. The animals were left to recover for the requisite post-operative period. Whether or not adrenalectomy had been completed was checked by measurement of plasma corticosterone concentrations and by inspection for possible adrenal remnants at autopsy.

2.2.5 Treatment of Blood Samples

Blood was collected with heparinised syringes (1000 I.U./ml

saline) into small plastic tubes (L.P.2: Luckham Ltd., Sussex) and kept on ice. Aprotinin (Trasylol, Beyer, Newbury, Berkshire) was added to samples at 1000 Kallikrein Inactivator Units (KIU)/ml blood. Samples were centrifuged at 1,720 x g for 20 min at 4°C, plasma was removed with a Pasteur pipette into plastic storage tubes (PT0944; Luckham Ltd.) and stored at -40°C.

2.2.6 Collection of Hypophysial Portal Blood

Hypophysial portal blood was collected according to the method of Worthington (1966) as used in this laboratory by Fink & Jamieson (1976) with some modifications.

The animal was anaesthetised with either urethane or sagatal and immobilised supine on an operating board. General anaesthesia was supplemented with 2% xylocaine (w/v) (Astra Pharmaceuticals, Watford, Herts) injected into the skin and muscle of the lower jaw. A midline incision was made in the skin of the lower jaw, extending caudally from the lower lip for 4-5cm. The muscles overlying the trachea were exposed by gentle retraction of skin and subcutaneous tissue. The trachea was exposed by making a 1cm longitudinal midline incision in the overlying muscles. A silk suture was passed beneath the trachea and a small transverse incision (rostral to the thread) was made in the trachea to allow the insertion of a piece of polythene tubing approximately 1.5cm in length (2.08mm o.d., 1.57mm i.d.). The tubing was secured by the silk suture. Gentle traction was applied to the tongue by passing a long silk Guy suture through the tip. Ligatures were placed round each mandibular ramus to prevent bleeding from branches of the lingual arteries taking care not to damage the tongue. The lower

jaw and floor of the mouth were then divided with a midline incision. The incision was extended through the muscles of the mouth floor parallel to each side of the mandible. The fauces were then cut in order to mobilise the tongue. Long sutures were placed through each of the flaps of the divided lower lip and were used for lateral retraction of the two sides of the jaw, the tongue was gently retracted using the Guy suture to reveal the soft palate and epiglottis. The tongue was then fixed over the chest under gentle traction. Sterilised cellulose gauze (Ethicon, Edinburgh) was used to prevent capillary and small vessel haemorrhage. All further manipulations were carried out under a Zeiss binocular operating microscope.

The soft palate was incised longitudinally along the midline with an iris electrocautery (Weiss & Son, London) from the posterior edge of the hard palate to 1mm anterior to the epiglottis and laterally to the pterygoid processes of the sphenoid bone. This exposed the mucosa overlying the basosphenoid which was then removed with cotton wool.

Using a dental drill, a hole was drilled in the outer table of the basisphenoid bone from the occipito-sphenoid suture to the basisphenoido-presphenoid suture anteriorly. Throughout the drilling procedure, the drill hole was packed with bone wax, (Ethicon Ltd., Edinburgh) especially in the area of the transverse venous sinus in the sphenoid bone. The exposed inner table was gently eburnated until it yielded to gentle pressure from either a fine drill or a pair of watchmaker forceps. The thin bone layer remaining was removed with watch maker's forceps revealing the dura mater overlying the median eminence and pituitary gland. A piece

of razor blade held in a pin chuck was used to make a "V" shaped cut in the dura mater. The base of the "V" was over the pituitary gland and the apex just rostral to the median eminence. Cerebrospinal fluid appearing at this stage was removed with cotton wool. The flap of cut dura was retracted caudally exposing the median eminence and rostral part of the anterior pituitary gland.

Animals were then injected with 2500IU heparin (heparin sodium 5000 IU/ml; Weddel Pharmaceuticals Ltd., London) into the external jugular vein. The pituitary stalk was cut transversely using fine iridectomy scissors (Weiss & Son, London) at its junction with the pituitary gland. Blood was allowed to flow into the drilled trough and was collected using a glass pasteur pipette. Fluid collected during the first 5 min was discarded to allow removal of tissue debris, bone wax or cerebrospinal fluid. Blood was then collected into ice cooled plastic tubes containing Trasylol. Peripheral samples were taken from the external jugular vein at the beginning of the collection period. Plasma was stored at -40°C until required for assay.

2.2.7 Median Eminence Stimulation

Animals were prepared as for collection of hypohysial portal blood until the median eminence and pituitary gland were exposed. Unipolar electrodes were made according to Jamieson & Fink (1976) in the Department of Pharmacology workshop. A 13mm piece of platinum wire (0.125mm diameter) was insulated with glass tubing to within 0.3mm of the tip and soldered to a length of copper wire. The whole assembly was mounted in a Teflon jig and fixed in place with acrylic cement (Simplex; Dental Fillings Ltd., London). The

electrode was lowered into position on the median eminence with the aid of a dissecting microscope and a micromanipulator. The electrode was placed on the median eminence, the portal vessels were cut and blood collected as described. Electrical stimulation was applied during the second half of the collecting period, using a constant current generator (Digitimer - Neurolog; Welwyn Garden City) based on parameters described by Jamieson & Fink (1976). The stimulus consisted of accurately balanced biphasic rectangular waves with a frequency of 50Hz, pulse width 1msec and amplitude of 1mA. The stimulus was applied in trains of 30 sec on and 30 sec off for a period of 30 min and were monitored on a calibrated oscilloscope.

2.2.8 Electrode Implantation

Bipolar electrodes were constructed as described in 2.2.7 with electrode tips separated by 1.0mm.

For implantation of stimulating electrodes, female rats (200-250g body weight) were anaethetised with tribromoethanol and positioned with the head immobilised in a stereotaxic frame. A midline incision was made in the scalp to expose the frontal and parietal bones, the periosteum was scraped off and a hole (4 x 4mm) drilled in the frontal bones immediately anterior to the bregma in order to expose the dura mater. Care was taken to avoid damage to the superior sagittal sinus. Bleeding during any point of the operation procedure was controlled with gentle pressure with cotton wool soaked in 0.9% saline solution. Three small stainless steel screws (approximately 1.5mm in diameter) were driven into small drill holes made around the outside margin of the exposed area. The dura mater was carefully incised in a longitudinal direction and

the electrode implanted according to the co-ordinates of de Groot (1959) and with the aid of a triplanar manipulator (Prior U.K.). Co-ordinates used are shown in Table 2.1.

Table 2.1

Area to be Stimulated	Co-ordinates Used (mm) Lateral	Anteroposterior	Ventral
PVN	0	6.4	3.4 - 3.8
Amygdala	4 - 4.5	5.4	2.5
Ventral Hippocampus	3.5 - 4.5	3.0	1.8

The electrode assembly was secured to the skull using dental cement which was carefully applied round the electrode hub and the screws. Once the cement had firmly set, the animal was removed from the stereotaxic frame and the scalp sutured. Animals were used for portal blood collection 7-10 days after the electrodes had been implanted. Parameters for electrical stimulation were as for median eminence stimulation (2.2.7).

2.3 RADIOIMMUNOASSAY OF CORTICOTROPHIN-RELEASING HORMONE

2.3.1 Introduction

Techniques for the measurement of physiological concentrations of biologically active substances require accurate and precise quantitation of picomole (10^{-12} mole) amounts of these compounds in biological fluids. The technique of radioimmunoassay (RIA), first described by Yalow (1959), is one of the most satisfactory methods of detection. Radioimmunoassay is highly sensitive, relatively simple and depending on the antiserum, highly specific.

The principles of RIA have been discussed in numerous reviews (Kirkham & Hunter, 1971; Yalow & Bersan, 1971; Jaffe & Behrman, 1979; Yalow, 1980). Briefly, a fixed concentration of labelled antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limiting; for example, only 30% of the total label concentration may be bound by the antibody. If unlabelled antigen is added to the system, there is competition for the constant and limited number of binding sites on the antibody. As the concentration of unlabelled antigen increases, the amount of labelled antigen bound will decrease. After equilibration between bound and free antigen has been achieved, the amount of antigen bound can be measured by separating antibody bound from free label by double antibody precipitation and counting. A standard curve is set up with increasing concentrations of standard unlabelled antigen and by comparison of displacement of labelled antigen, the amount of antigen in unknown samples can be calculated.

Each standard curve contains tubes with only labelled antigen (total counts, T.C.) and tubes without antiserum to assess non-specific binding (NSB), the number of counts trapped nonspecifically in the precipitate. The total bound (Bo) is the amount of labelled antigen which binds at a given concentration of antiserum when no unlabelled antigen is present.

Many radioimmunoassays have been described for ovine-CRH (Gibbs & Vale, 1982; Linton & Lowry, 1982) and used to determine concentrations of rat and human CRH in plasma and hypothalamic samples. However, the isolation and characterisation of a 41 amino acid rat hypothalamic peptide with potent corticotropin releasing

factor activity (Rivier et al. 1983a) has made it possible to develop a more specific RIA for the measurement of CRH in rat samples.

2.3.2 Preparation of Antiserum to CRH

Antiserum is prepared by injecting the antigen, usually in an oil/water emulsion containing an adjuvant into a suitable animal. This is usually a rabbit, although sheep, guinea pigs or donkeys are sometimes used.

300µg of rat CRH (Peninsula) was dissolved in 0.5ml of saline and emulsified with an equal volume of Freunds' complete adjuvent. The immunogen was administered by multiple intradermal injections into three New Zealand white rabbits. At the same time, the animals were given a single subcutaneous injection of 0.5ml B. pertussis vaccine (Difco, Detroit, MI). At six week intervals, the animals were boosted with further injections of 200µg of the immunogen in Freund's complete adjuvant and bled from the ear vein at 10-14 day intervals after each booster injection. The antiserum used for radioimmunoassay was obtained from a single rabbit 10 days after the fourth booster dose of rCRH was administered and will be referred to as GF 22*4.

At about the same time, a commercial antiserum to human CRH (rabbit anti rCRH) became available and was purchased from IgG Corporation (Nashville, TN). IgG antisera was also tested in the RIA for rCRH. The antisera, purchased for 1,000 tubes, was dissolved in 1.0ml phosphate-EDTA buffer and stored in 100µl aliquots at -40°C.

2.3.3 Iodination

A variety of methods are available for the radioiodination of peptides and proteins. Iodination methods can be broadly divided, direct methods in which ^{125}I is directly incorporated into tyrosine and/or histidine residues, and conjugation methods in which a radioiodinated moiety is conjugated to a specific side chain, mostly NH_2 groups on side chains and at the N-terminus. Conjugation methods are more complex but can be used to label peptides that do not contain tyrosine.

The most widely used method of iodination is the chloramine-T oxidation of Na ^{125}I in the presence of the protein or peptide to be labelled (Hunter & Greenwood, 1962). This results in incorporation of ^{125}I into the tyrosine residues in high yield. Excess chloramine-T is reduced by the addition of a reducing agent such as sodium metabisulphite. In order to overcome the problems of peptide damage caused by soluble oxidising agents such as chloramine-T, Fraker & Speck (1978), examined the use of the insoluble oxidising agent 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoturil (Iodogen). Iodogen is coated onto the surface of the reaction vessel and iodination effected by the addition of Na ^{125}I and peptide solution. A gentle method for the iodination of peptides using the enzyme lactoperoxidase to catalyse the oxidation of iodide in the presence of small amounts of hydrogen peroxidase was first described by Marchaloanis (1969). The reaction is initiated by the addition of hydrogen peroxide and terminated by the addition of cysteine or by dilution.

Four methods of iodination of CRH were tested and are described below.

2.3.3.1 Conjugation Method

The most commonly used conjugation reagent is N-succinimidyl 3-(4-hydroxy 5-[^{125}I] iodophenyl) proprionate (Bolton & Hunter reagent; Bolton & Hunter, 1973) and was, therefore, used to iodinate CRH. The reagent is conjugated to CRH under slightly alkaline conditions.

The reagents were:

r-CRH	5µg/10µl 0.1M Borate Buffer pH 8.5
Bolton Hunter Reagent	1mCi Amersham 1M 5861
Glycine	0.2M in 0.1M Borate Buffer pH 8.5
Trifluoroacetic acid/ Potassium Iodide (TFA/KI)	10mg ml^{-1} KI in 0.2% (v/v) TFA

Method: 1mCi Bolton Hunter reagent was dried down under nitrogen and 5µg r-CRH was added. After 15 min on ice, 500µl glycine was added and left for 5 min on ice before the reaction was terminated by the addition of 500µl TFA/KI.

2.3.3.2 Iodogen Method

The reagents were:

Tyr^{0}-CRH	0.2mg ml^{-1} in 0.05N HCl
Iodogen	40µg ml^{-1} dichloromethane
Na ^{125}I	Amersham IM530: 3.7 G Bq ml^{-1}
TFA/KI	10mg ml^{-1} KI in 0.2% (v/v) TFA

Method: 50µl of the stock solution of Iodogen was evaporated to dryness under nitrogen in a polypropylene tube. 2µg Tyr^{0}-CRH, 20µl 0.5M phosphate buffer and 10µl (1mCi) Na ^{125}I were added together and incubated for 10 min at room temperature. The reaction was terminated by the addition of 200µl 0.5M phosphate

buffer and 500µl TFA/KI.

2.3.3.3 Lactoperoxidase Method

The reagents were:

Tyr^0-CRH	0.2mg ml^{-1} in 0.05N HCl
Na ^{125}I	Amersham IMS 30: 3.7 G Bq Ml^{-1}
Lactoperoxidase	1mg ml^{-1} in 0.05M phosphate buffer
H_2O_2	0.03% solution
TFA/KI	10mg ml^{-1} KI in 0.2% (v/v) TFA.

Method: The peptide was placed in a polypropylene tube together with 25µl 0.5M phosphate buffer, 10µl Na ^{125}I (1mCi) and 10µl of stock lactoperoxidase. The reaction was initiated by the addition of 5µl 0.03% H_2O_2 and incubated for 120 sec at room temperature. The reaction was terminated by addition of 100µl horse serum, 100µl 0.5M phosphate buffer and 400µl TFA/KI.

2.3.3.4 Chloramine-T Method

The reagents were:

Tyr^0-CRH	0.2mg ml^{-1} in 0.05N HCl
Na ^{125}I	Amersham IMS 30: 3.7 G B& ml^{-1}
Chloramine-T	1mg ml^{-1} in 0.05M phosphate buffer
Sodium-Metabisulphite	2.5mg ml^{-1} in 0.05M phosphate buffer
TFA/KI	10mg ml^{-1} KI in 0.2% (v/v) TFA

Method: 10µl Tyr^0-CRH was placed in a polypropylene tube and 10µl 0.25M phosphate buffer, 10µl Na ^{125}I and 10µg of chloramine-T were added. After 20 sec the reaction was terminated by the addition of 25µg sodium metabisulphite, 180µl 0.25M phosphate buffer and 620µl TFA/KI.

The four crude iodination mixtures were purified as described below and tested in the assay for binding to antisera (Bo) and non-specific binding (NSB).

2.3.3.5 Purification of Labelled Antigen

Methods describing the purification of ^{125}I-Tyr^{0}-CRH from free label by reverse phase high performance liquid chromatography (HPLC) (Gibbs & Vale, 1982; Linton & Lowry, 1982) have been described. In this study ^{125}I-Tyr^{0}-CRH was purified from the crude iodination mixtures by HPLC (Harmar & Rosie, 1984). This system consists of a two-channel mini-pump (Milton Roy Co., Riviera Beach, FL) connected in series to a three way valve, a sample injection valve with a 1ml loop (Rheadyne Berkeley, LA) and a guard column holder (Brownlee MPLC cartridge system: Brownlee Labs, Santa Clara, CA) containing a disposable cartridge of bonded phase silica, 3cm x 0.46cm (Spheri 5 cyano: Brownlee Labs). The two channels of the pumps were primed respectively with (a) 0.2% (v/v) aqueous trifluoroacetic acid (TFA) and (b) 0.2% (v/v) TFA in methanol. After equilibration of the column in aqueous TFA, the reaction mixture was injected and eluted with a stepwise gradient of methanol/TFA in increments of 10% from 50-100%B. The flow rate was 1ml min^{-1} and six fractions of 1ml were collected at each step in the gradient. Free Na ^{125}I passed unretarded through the column and was eluted first. This method was used to purify crude mixtures from all four iodination methods.

2.3.3.6 Comparison of Results

The sequence of rat-CRH contains no tyrosine residues and,

therefore, initially conjugation to Bolton Hunter reagent was used. Purification of the labelled antigen resolved three peaks, only one of which was immunogenic. This label gave low total binding (Bo) and high NSB values, suggesting the antigen may be damaged. Tyr^{0}-CRH then became available commercially (Peninsula Laboratores Inc. CA) and more direct methods of iodination were accessible. Gentle methods of direct iodination, Iodogen and lactoperoxidase were found to be inconsistent in their incorporation of ^{125}I. Purification of the label resolved peaks in the same way as Bolton Hunter iodination with one peak being immunogenic. Again NSB values were high and Bo's quite low and, therefore, chloramine-T iodination was used. Gibbs & Vale (1982) have described the use of chloramine-T to iodinate ovine-CRH and this method was found to be very successful in the iodination of rCRH. Immunogenic, labelled CRH was eluted in 70% methanol/TFA with a specific activity of 250μCi/μg CRH, an elution profile is shown in Fig. 2.1. Non-specific binding was less than 2% and total binding was greater than those with other labels and was therefore the iodination method used in this study.

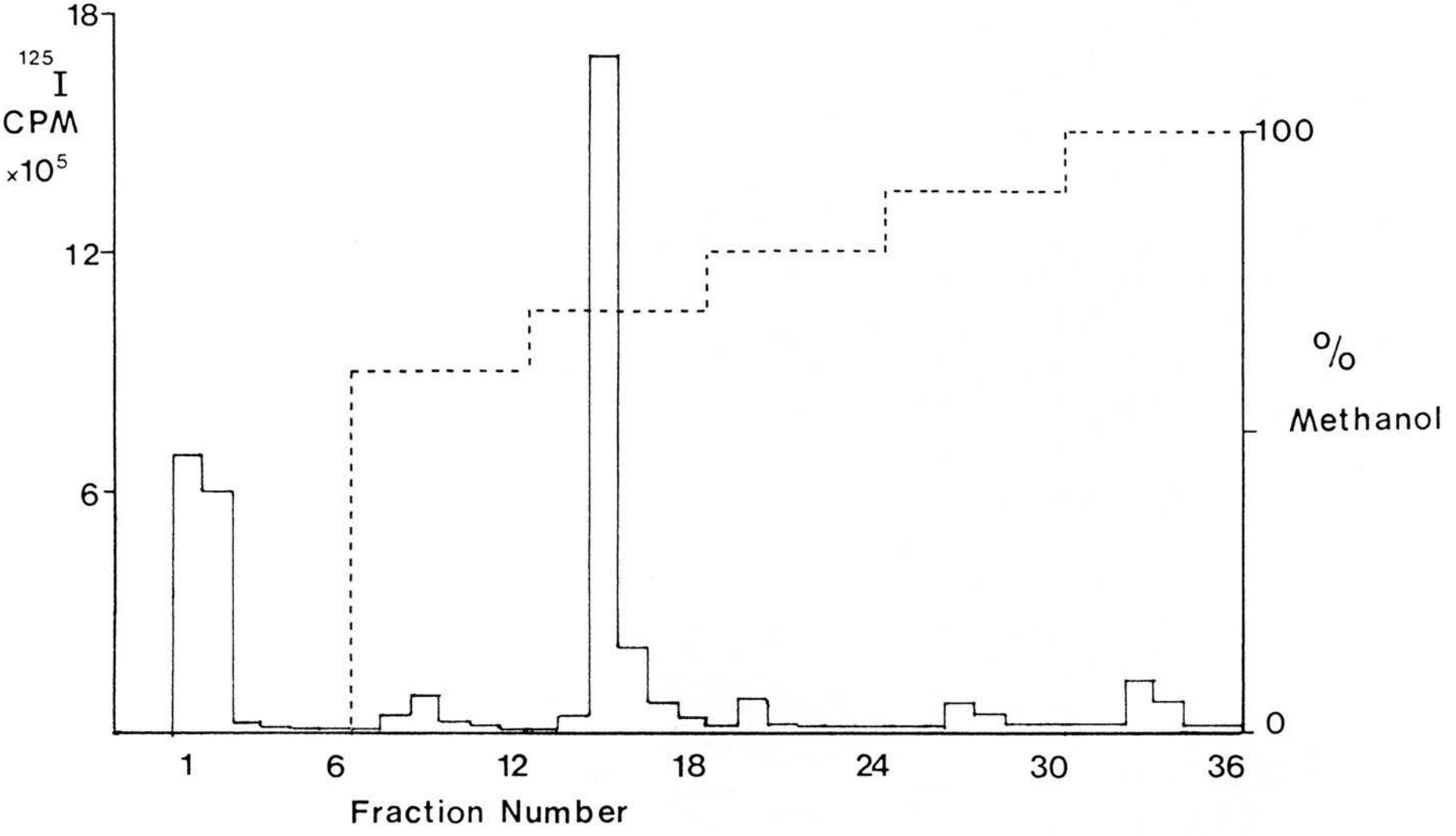
Labelled Tyr-CRH can be stored at -70°C for up to four weeks without decrease in immunogenicity. After this period, total binding is reduced and non-specific binding is increased. The addition of 2-mercaptoethanol did not increase the length of time over which the label retained its immunogenicity.

2.3.4 Optimisation of Assay Conditions

In order to determine suitable antisera dilutions to give maximum sensitivity, antiserum dilution curves were set up (Fig.

Figure 2.1 Isolation of ^{125}I-labelled Tyr^{O}-rCRH by liquid chromatography

Tyr^{O}-rCRH was iodinated and the reaction products were separated by liquid chromatography as described in section 2.3.3.5. The solid line shows c.p.m. per fraction and the broken line percent methanol in eluant.


18
125 I CPM ×10^5
12
6
100
%
Methanol
0
1
6
12
18
24
30
36
Fraction Number

2.2) with dilutions ranging from 1:25 to 1:200 for IgG anti-CRH and from 1:200 to 1:16,000 for GF22*4 anti CRH. Antiserum dilutions of 1:90 (final dilution 1:270) for IgG antiserum and 1:4,000 (final dilution 1:12,000) for GF22*4 gave total binding (Bo) values of 20-25% and were used in all further studies.

It is well established that different buffers, separation techniques and time course can be altered to optimise conditions for RIA. In the present study, these conditions were altered to increase sensitivity of the assay.

The optimum pH for antisera binding in RIA is generally 7-7.5 and, therefore, all buffers tested, were in this range. Triton X-100 was added to some buffers to reduce adsorption of CRH to assay tubes and, therefore, reduce non-specific binding. Different incubation times were also tested for binding of antibody, non-specific binding and sensitivity of the assay.

2.3.4.1 Buffers

Composition of standard buffers is in Appendix I. The buffers tested were:

(1) RIA Standard Diluent

a) Phosphate-EDTA pH 7.4 + 0.1% Triton X-100

b) Phosphate-EDTA pH 7.4 + 1% BSA + 0.1% Triton X-100

c) Phosphate-EDTA pH 7.4 + 0.03% Triton X-100

d) Phosphate-EDTA pH 7.4 + 0.03% Triton X-100 + 1% BSA

Antiserum Buffer

All the above buffers were 1% (v/v) with Normal Rabbit Serum (NRS) and were used in the assay with their respective RIA standard diluent.

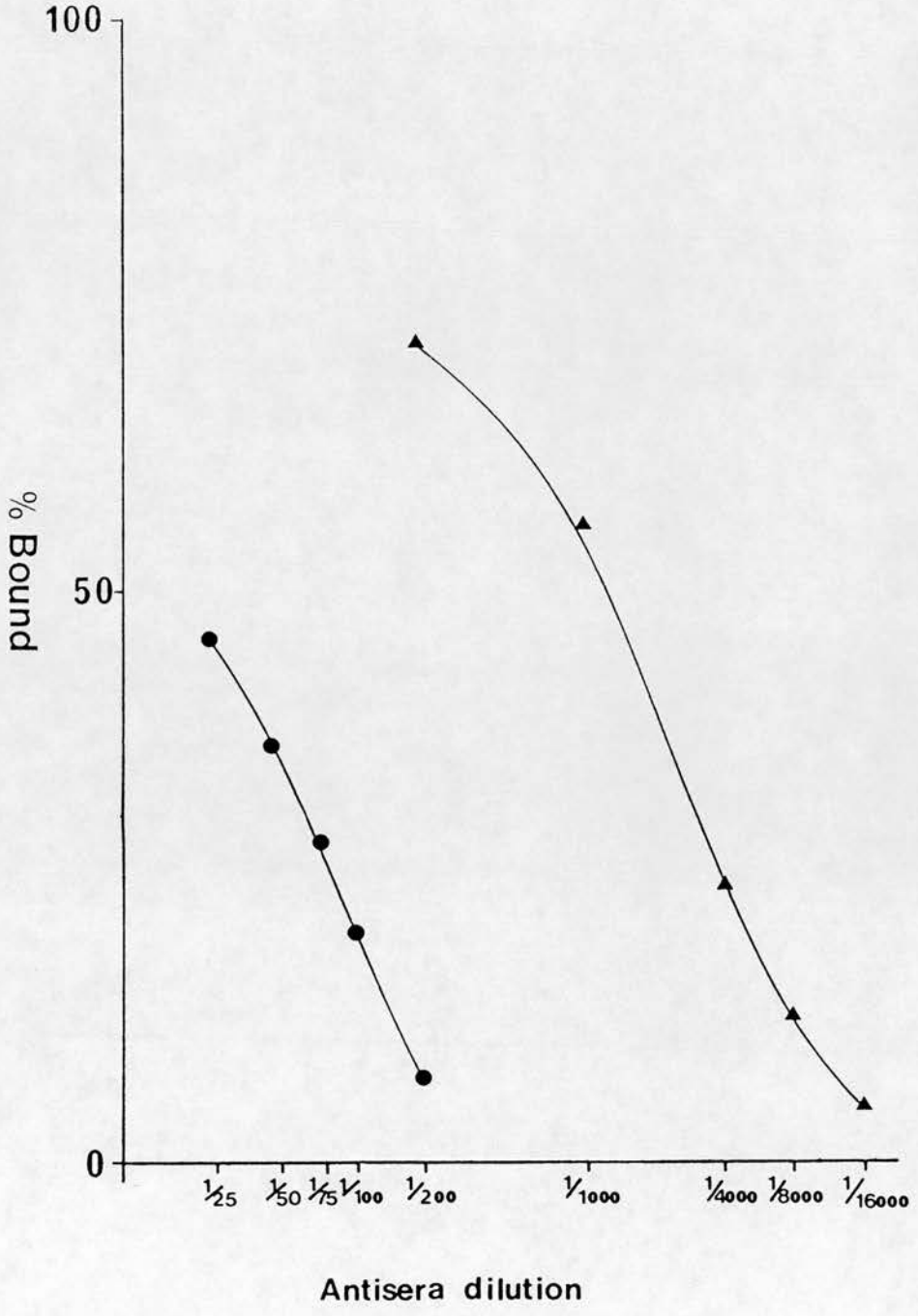


Figure 2.2 Binding of ^{125}I-Tyr^{0}-CRH at increasing antisera dilutions

^{125}I-Tyr^{0}-CRH binding was reduced as the dilution of IgG anti CRH serum (●) and GF22*4 anti CRH serum (▲) was reduced. 20-25% binding was obtained at a dilution of 1:90 for IgG antiserum and 1:4000 for GF22*4 antiserum.

2. RIA Standard Diluent

PBS pH 7.4 + 1% BSA + 200 KIU/ml Trasylol

Antiserum Buffer

a) PBS/NRS + 200 KIU/ml Trasylol

b) PBS/NRS + 2000 KIU/ml/Trasylol

Both antiserum buffers were used in conjunction with RIA standard diluent (2)

All the buffers were initially tested for NSB and Bo values. Buffers 1a), 1c) and 2b) gave similar binding which were 2- to 4-fold greater than the other buffers. The reduction from 0.1 to 0.03% in Triton X-100 in buffer 1c) did not affect the NSB values and made the pellet more stable, therefore, decreasing assay variability. Buffers 1c), and 2b) were then tested in a standard curve to establish which one gave the most sensitive assay.

Standard curves were constructed using rat-CRH (Peninsula Labs Inc.). A stock solution at 1μg/μl in 0.05N HCl was diluted in the different buffers and in stripped plasma to concentrations of 10, 20, 50, 100, 200, 500 and 1000pg/ml. Aliquots of 100μl were used and stored at -40°C until required. Both buffer standard curves were tested with the two antisera 1) IgG anti-CRH at an initial dilution of 1/90 and 2) GF22*4 at an initial dilution of 1/4,000. As can be seen in Fig. 2.3, buffer 1c) gave a standard curve that was 2-fold more sensitive than the buffer 2b) curve using IgG antiserum and 4-fold more sensitive when using GF22*4 antiserum. Therefore, IgG antiserum was used routinely with buffer 1c) in the RIA for rat CRH.

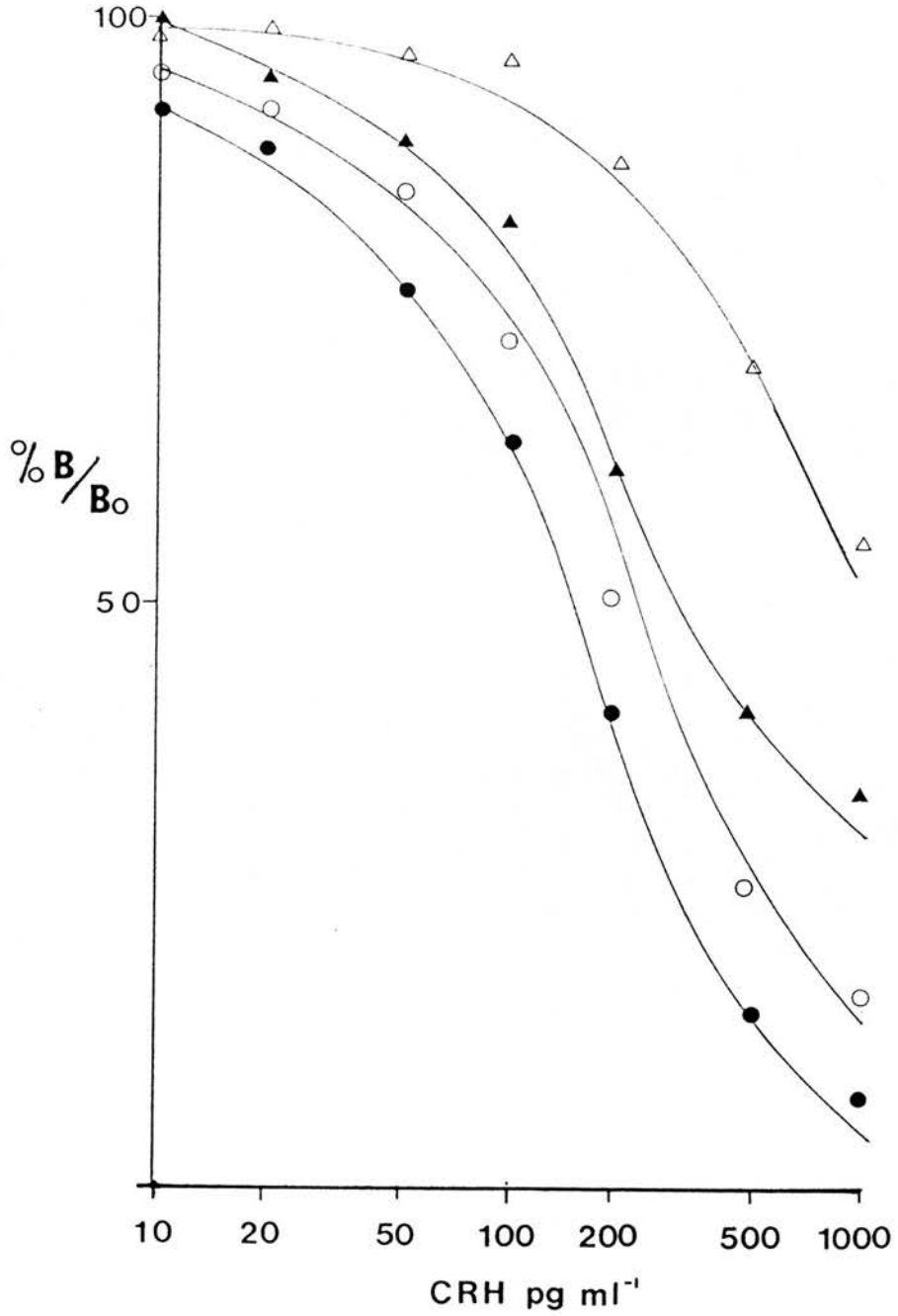


Figure 2.3 Standard curves for CRH using different buffers and antisera

Standard curves were set up with standards of rCRH ranging from 10-1000pg ml^{-1} in 100µl of RIA standard diluent. The buffers tested were phosphate-EDTA pH 7.4 + 0.03% Triton X-100 (closed symbols) and PBS + 1% BSA + 200 KIU/ml Trasylol (open symbols). Both buffers were tested with IgG antiserum, 1:90 (● ○) and GF22*4, 1:4000 (▲ △).

2.3.4.2 Separation

The most common method of separation of antibody bound label from unbound label and standard unlabelled material is by precipitation with an antibody against gamma globulins, in this case, Donkey anti-rabbit gamma globulins (ARGG). Following incubation with ARGG, the double antibody complex is precipitated. Separation can also be achieved by dextran-coated charcoal, alcohol precipitation or precipitation with Staphylococcus aureus. In this study, different concentrations of ARGG (SAPU) were examined for levels of binding (Bo) and variability. Final dilutions of ARGG tested were 1/30, 1/60 and 1/120. Pellet stability was further increased by the addition of 1.6ml washing buffer (Phosphate-EDTA pH 7.4 + 2.5% BSA) before centrifugation.

ARGG concentration	% Binding ± S.E. (n = 6)
1:30	35.5 ± 7.3
1:60	38.8 ± 4.7
1:120	5.2 ± 24.2

Optimum conditions for precipitation were obtained with ARGG in a final dilution of 1:60.

2.3.4.3 Time Course

Incubation conditions were checked in order to establish the optimum time course for maximum sensitivity. Standard curves were set up for three different time courses and the incubation times were as described below. All incubations were at 4°C.

	1	2	3
Day 1	Antibody + Label	Antibody	Antibody
2	ARGG	-	-
3	Wash + spin	Label	-
4		ARGG	Label
5		Wash + spin	-
6			ARGG
7			Wash + spin

Standard curves obtained are shown in Fig. 2.4. From these, it can be seen that an increase in the incubation time of antiserum with standard antigen increased the sensitivity of the assay. Therefore, the seven day assay was used routinely for rat CRH.

2.3.4.4 Optimal Conditions for RIA of r-CRH

On the basis of these results, the optimal conditions for RIA of rCRH were as follows:

1) Assay Buffer: Phosphate-EDTA pH 7.4 + 0.03% Triton X-100
2) Antiserum Buffer: Assay Buffer + 1% NRS containing IgG antiserum at an initial dilution of 1:90
3) Double antibody: Assay Buffer + ARGG (SAPU) at 1:15 initial dilution
4) Incubation: 7 day assay

These conditions gave an RIA for CRH with a sensitivity of 2-4pg per tube.

2.3.5 Plasma Extraction

CRH is present in very low concentrations in plasma, therefore, large volumes of plasma must be included in the assay to allow

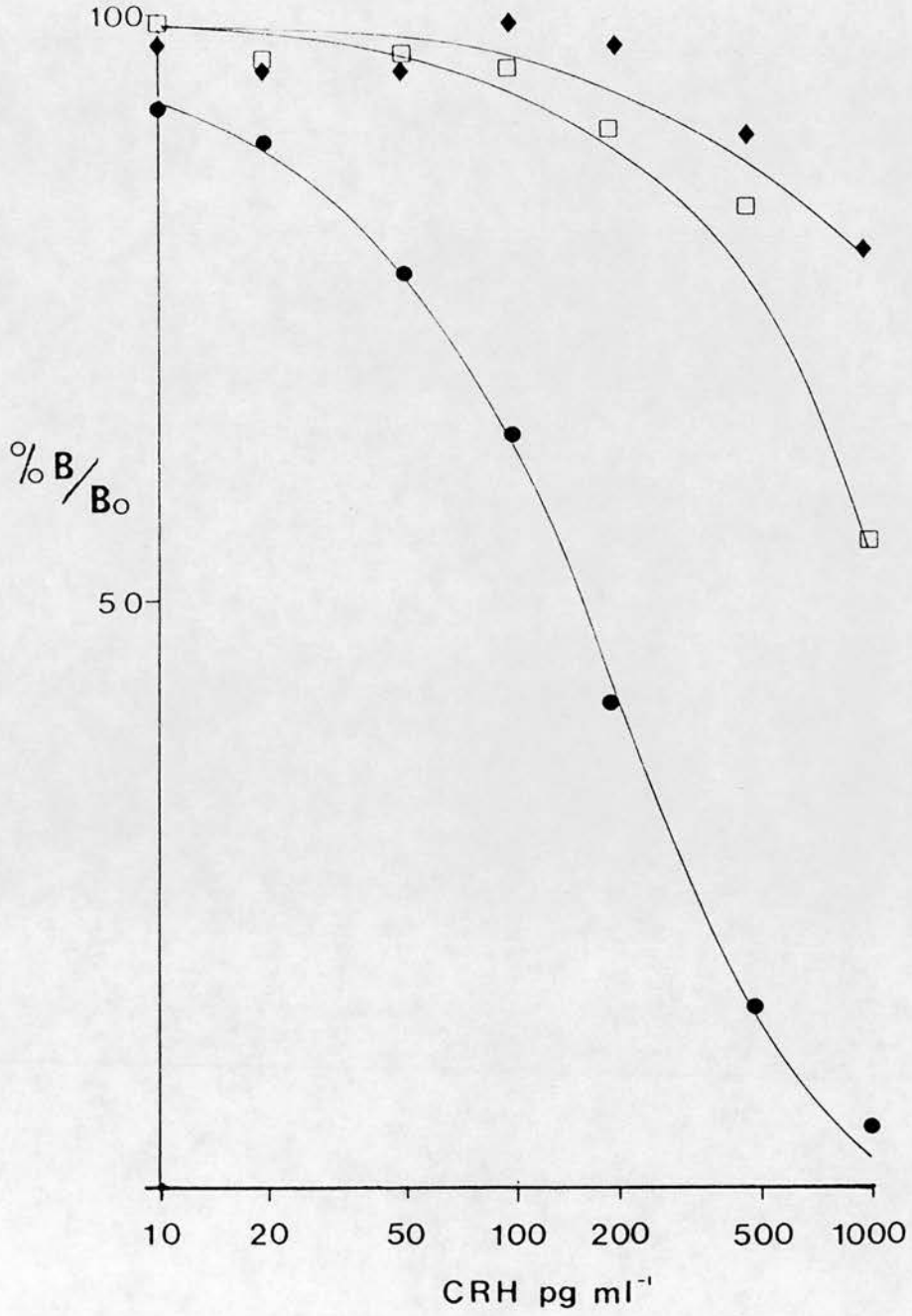


Figure 2.4 Standard curves for CRH with different incubation times

Standard curves were set up with standards of rCRH using three different incubation times (◆) - 3 day (□) - 5 day and (●) - 7 day assay as described in 2.3.4.4.

detection of CRH present. However, the introduction of plasma to the assay reduced total binding (Bo) and construction of a standard curve was impossible. It was necessary to extract CRH from large volumes of plasma by removing plasma proteins and salt thereby making the sample suitable for RIA.

Many methods extract CRH from plasma by adsorption onto silicic acid. This method relies on the hydrophobic properties of peptides such as CRH. Plasma proteins and salts are washed off the silicic acid by a water-based solvent and the peptide eluted with an organic solvent. Silicic acid is available as ODS silica cartridges (Bond Elut, Scotlab, Carluke) or as 100-200 mesh silicic acid (Sigma Chemicals). A simpler method of extraction of peptides from plasma is the precipitation of plasma proteins by methanol leaving peptides in the supernatant. These three methods of extraction were tested for recovery of rCRH from plasma samples.

2.3.5.1 Silicic Acid Extraction

Plasma samples of 2.4ml (or samples made up to 2.4ml with hormone free plasma) were mixed with 1ml of silicicacid slurry (200mg/ml) in phosphate-EDTA buffer. The samples were then mixed, end over end, at room temperature for 30 min and centrifuged at 1720 x g for 10 min. The resultant pellet was resuspended in 2.5ml H_2O by vortexing and the sample centrifuged as before. The supernatant was discarded and peptides eluted from the silicic acid pellet with a solution of acetone:acetic acid:water in a ratio of 20:1:79. The pellet was resuspended in 2.5ml of elution solvent and mixed, end over end, for 30 min before centrifuging as above. The supernatant was collected and the elution procedure repeated.

The resulting 5ml of supernatant was freeze dried and then resuspended in assay buffer. Standards and blanks were made up in plasma and extracted as described above.

2.3.5.2 ODS Silica Cartridges

"Bond Elute" cartridges (100mg sorbent, 1ml reservoir: Scotlab, Carluke) were prepared by washing with 2ml methanol/1% (v/v) HCl, solvent (A) followed by 2ml 1% (v/v) HCl, solvent (B). Plasma samples were acidified with 5 volumes of solvent (A) and applied to the cartridges. After two 1ml washes with solvent (A), peptides were eluted with two applications of 1ml solvent (B). The eluate was evaporated to dryness under vacuum and the extract was reconstituted in assay buffer. Plasma standard curves for CRH and hormone free plasma were extracted as above. The same system was also used with different solvents (A) 75% acetonitrile, 25% 50mM Triethylammonium formate (TEAF) pH 3.0 (B) TEAF pH 3.0.

2.3.5.3 Methanol

Plasma proteins were precipitated in plasma samples and standards by addition of ice-cold methanol to a final concentration of 85% methanol. The samples were mixed and left on ice for 10 min. Proteins were precipitated by centrifugation at 4°C, 1,720 x g for 20 min. Supernatants were dried under vacuum, the extracts reconstituted in assay buffer and transferred to fresh assay tubes.

2.3.5.4 Comparison of Methods

Recovery of CRH from ODS silica cartridges was not consistent and calculation of a standard curve impossible. Standard curves

from methanol extraction and silicic acid extraction are shown in Fig. 2.5. Both extraction techniques gave standard curves that were parallel with the unextracted buffer curve but the recovery of standard was greater from methanol extraction. Detection limits for methanol and silicic acid were 2-4 and 6-8pg per tube, respectively. The large volumes of plasma required for silicic acid extraction made it impractical for the small volumes of portal blood available for assay. Therefore, the recovery, adaptability and simplicity of the methanol extraction made it the extraction method used for all plasma samples to be assayed for CRH.

2.3.6 Assay Operation

2.3.6.1 Sample Preparation

Plasma samples (25-100µl) were extracted with 85% methanol as described in 2.3.5, dried down overnight and reconstituted in 100µl assay buffer. The samples were transferred to fresh tubes for RIA.

Tissue samples were homogenised in 5-10 volumes of 2M acetic acid, diluted in 2M acetic acid and an aliquot taken for RIA. Each aliquot was dried down and reconstituted in 100µl assay buffer. Inhibition of binding by serial dilution of portal plasma extracts was parallel with the standard curve for CRH (Fig. 2.6).

2.3.6.2 Assay Conditions used in Experimental Studies

The following assay procedure was used, all incubations were at 4°C.

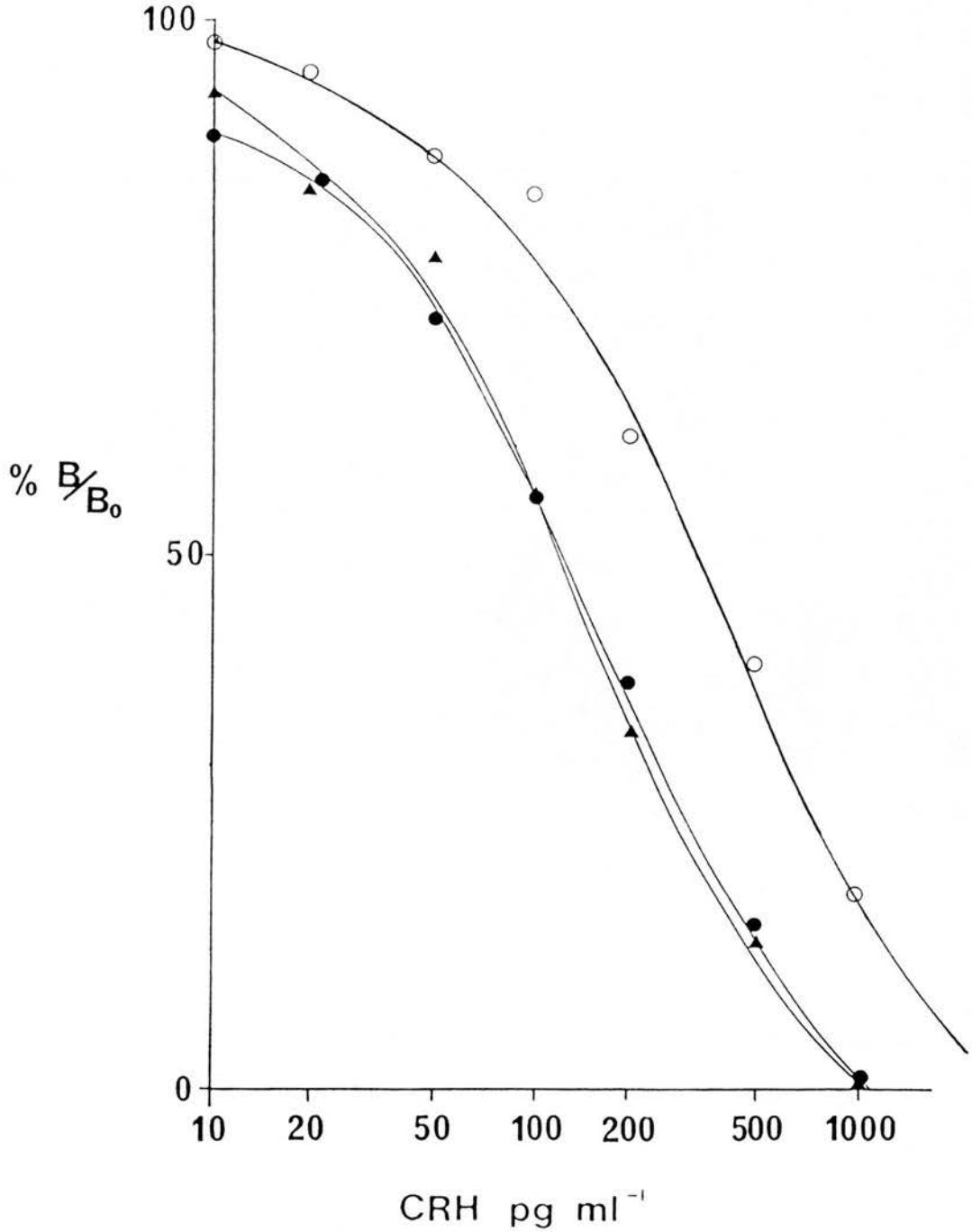


Figure 2.5 Comparison of methods for the extraction of rCRH from plasma

Standard curves for rCRH were set up in RIA standard diluent (●) and compared with plasma standard curves for rCRH that had been extracted with 85% methanol (▲) and with silicic acid (○).

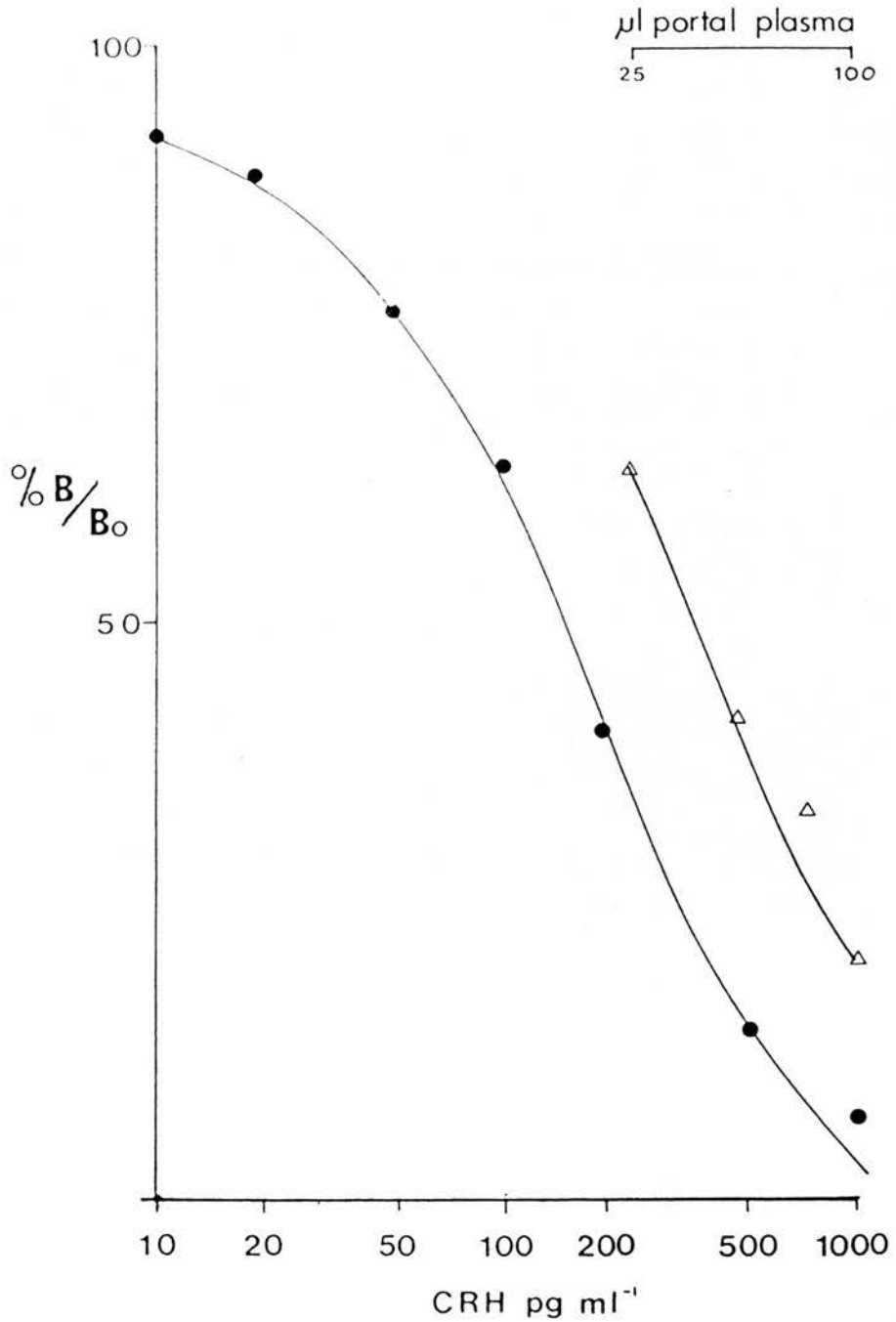


Figure 2.6 Comparison of displacement curves produced by synthetic rCRH and dilutions of extracted hypophysial portal plasma

Extracted plasma standard curves were set up (●) and compared with the displacement curve produced by extracts of 25–200µl hypophysial portal plasma (△). Extracts of rat portal plasma showed parallelism with standard rCRH.

Day 0 Plasma samples, standards and pools were extracted with 85% methanol and the supernatants evaporated under vacuum overnight. Standards, NSBs, Bo and pools were extracted in triplicate.

Day 1 The samples were reconstituted in 100µl assay buffer (phosphate-EDTA pH 7.4 + 0.03% Triton X-100), T.C., NSB, Bo and standards were made up in triplicate. All assay tubes except TC and NSB tubes were mixed with 100µl antiserum buffer (assay buffer + 1% NRS) containing IgG-anti CRH at an initial dilution of 1:90.

Day 4 100µl assay buffer containing 5,000cpm ^{125}I-Tyr^{0}-CRH, iodinated by chloramine-T and purified by HPLC was added to the tubes and the tubes were mixed.

Day 6 100µl assay buffer containing 1:15 ARGG (SAPU) was added to the tubes and mixed.

Day 7 The tubes were washed with 1.6ml phosphate-EDTA pH 7.4 + 2.5% (w/v) BSA.

All tubes except T.C. were spun for 30 min at 4°C, 1,720 x g. Supernatants were aspirated and pellets counted for 3 min in a Berthold gamma counter (MAG 315). The data were analysed by an on-line computer which constructed a standard curve using a log logit transformation and also gave the concentrations of CRH in unknown samples. The assay cut off point was at 10% and 90% B/Bo.

2.3.6.3 Quality Control

Pools of r-CRH in rat plasma at low and high concentrations were stored in aliquots of 100µl at -40°C. Both low and high pools were

included in the extraction procedure for each assay. Intra-assay variability for low and high pools were 10.9% and 3.4% respectively. Inter-assay variability for low and high pools were 13.5% and 9.4% respectively.

2.3.6.4 Hormonal Specificity

Various peptide hormones were tested to determine antibody specificity for both antisera used. Dynorphin, β-endorphin, luteinising hormone releasing hormone, somatostatin-14, adrenocorticotrophin hormone (ACTH), thyrotrophin releasing hormone, sauvagine and ovine-CRH were tested in the assay, crossreactivity at concentrations of up to 1μg/ml is shown in Fig. 2.7. Neither of the two antisera crossreacted more than 0.1% with any of the peptides.

2.3.6.5 Comparison with Other Assays

The assay for rCRH developed here for buffer and extracted plasma samples has a sensitivity of approximately 2-4pg/tube at the 90% B/Bo cut off point. These detection limits compare favourably with rCRH assays developed by Sasaki et al. (1984) and by Gibbs & Vale (1983) who report sensitivities of 3.5 and 10pg/tube respectively. Both these groups also found it necessary to extract large volumes of plasma to measure concentrations of CRH. Inhibition of binding by dilutions of plasma extracts was parallel with standard curves for rCRH.

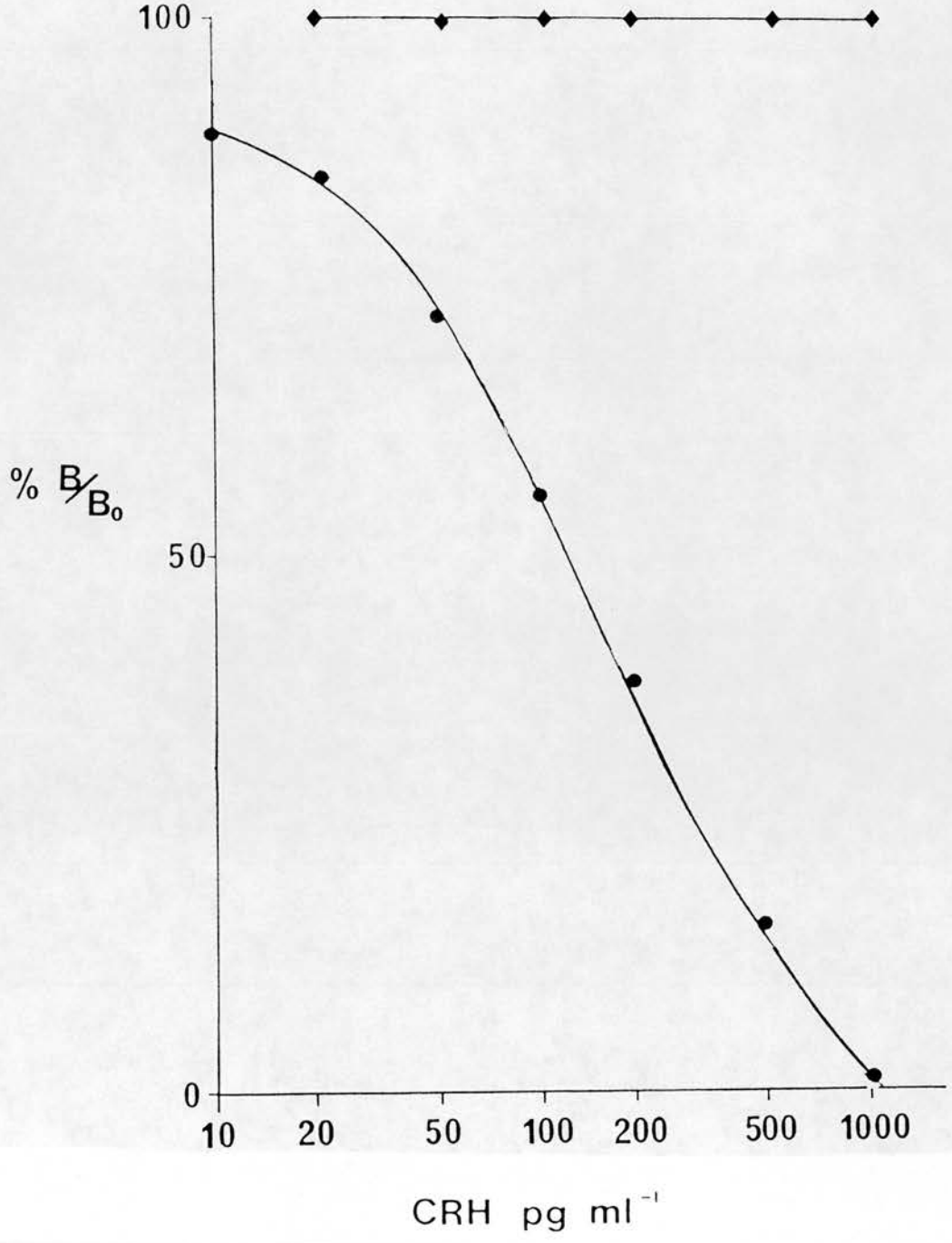


Figure 2.7 Crossreactivity of peptides with IgG CRH antiserum

Displacement curves for rCRH and other peptides are shown. No peptides other than rCRH crossreacted with the antiserum.
(●) rCRH
(◆) dynorphin, β-endorphin, luteinising hormone releasing hormone, somatostatin-14, ACTH, thyrotrophin releasing hormone, sauvagine and ovine-CRH.

2.4 RADIOIMMUNOASSAY FOR ACTH

2.4.1 RIA Standard Diluent

RIA standard diluent consisted of ACTH RIA buffer (Appendix I) made with 3.5% (w/v) BSA. The buffer was then extracted with silicic acid. Silicic acid (SIL-A-200, 60-200 mesh screen, Sigma) was added to RIA standard diluent at 100mg ml^{-1} and mixed end over end for 30 min at room temperature. The buffer was then centrifuged at 1,720 x g for 10 min and the supernatant removed and frozen at -40°C until required. Immediately before use, the buffer was thawed and Trasylol (250KIU/ml) and 0.03% (v/v) Triton X-100 were added.

2.4.2 Reference Standard

A standard curve was constructed using human ACTH (NIADDK) made up to 5, 10, 20, 50, 100, 200 and 500pg/ml in RIA standard diluent. The standards were stored at -40°C in 100µl aliquots until required.

2.4.3 First Antibody

IgG-ACTH-1*(IgG Corporation, Nashville, TN) was diluted 1:10,000 in RIA standard diluent made 1% (v/v) with normal rabbit serum.

2.4.4 Labelled Antigen

Reagents used were:

Na ^{125}I	Amersham IMS 30: 3.7 GBq ml^{-1}
Human ACTH	0.2mg ml^{-1} in 0.01M HCl
Chloramine-T	1mg ml^{-1} in 0.05M Phosphate buffer
Sodium Metabisulphite	2.5mg ml^{-1} in 0.05M Phosphate buffer
KI/TFA	10mg ml^{-1} KI in 0.2% (v/v) TFA

Method: 10µl human ACTH was placed in a polypropylene tube and 10µl 0.25M phosphate buffer, 10µl Na ^{125}I and 10µl chloramine-T were added. After 20 sec, the reaction was terminated by the addition of 10µl sodium metabisulphite, 180µl 0.25M phosphate buffer and 620µl KI/TFA. Purification of labelled antigen from the crude iodination mixture was carried out as described in 2.3.3. Labelled immunoreactive ACTH eluted in 60% methanol and was stored at -70°C for up to four weeks.

2.4.5 Second Antibody

Donkey ARGG (SAPU) was diluted 1:5 in RIA standard diluent.

2.4.6 Separation Buffer

The separation buffer used was ACTH RIA Buffer containing 2.5% (w/v) BSA.

2.4.7 Assay Procedure

The following assay procedure was used:

Day 1	100µl of standard or sample was added to 100µl IgG-ACTH-1 diluted 1:10,000 in standard diluent. The tubes were mixed and incubated overnight at room temperature.
Day 2 a.m.	100µl ^{125}-I-ACTH-containing 10,000cpm in standard diluent was added and the tubes were mixed and left at room temperature for 8h.
p.m.	100µl ARGG 1:5 in standard diluent was added. The tubes were mixed and incubated overnight at 4°C.

Day 3 1.6ml of separation buffer was added to each tube and the tubes immediately centrifuged at 1,720 x g for 30 min at 4°C. The supernatant was aspirated and the pellet counted. A standard curve was calculated as described in 2.3.6.2 and a representative curve is shown in Fig. 2.8.

2.4.8 Quality Control

Pools of ACTH in rat plasma were stored in aliquots of 100µl at -40°C and were included in each assay. Intra- and inter-assay variation were 13.4% and 5.4% respectively.

2.5 RADIOIMMUNOASSAY FOR CORTICOSTERONE

2.5.1 Radioimmunoassay Buffer

The buffer used in this assay was PBS 0.1% Gelatin pH 7.0

2.5.2 Purification of [1,2,6,7^{3}H] Corticosterone

100µl (11.1µCi) of stock [1,2,6,7,^{3}H] corticosterone was evaporated to dryness under nitrogen and redissolved in 40µl of dichloromethane. This was spotted onto Instant Thin Layer Chromatography S.A.F. paper (Gelman, Hexely) and run against 5µg cold corticosterone, the solvent used was eight parts chloroform to one part acetone The position of the cold corticosterone was detected by spraying the paper with 2,4-DNPH/HCl which turns yellow/orange on contact with ketones. The corresponding area for the labelled antigen was excised and eluted with 5mls acetone After

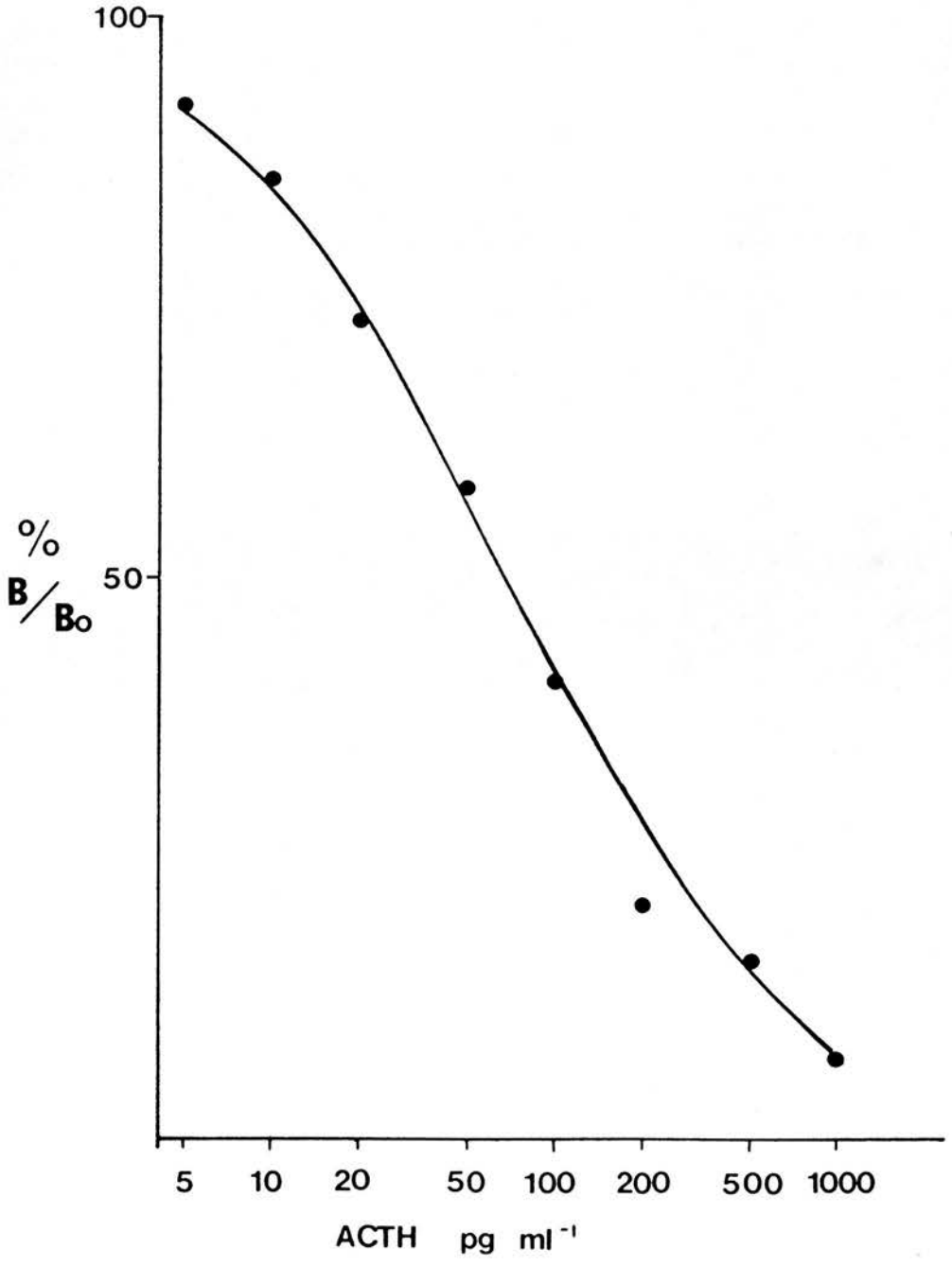


Figure 2.8 Standard curve for ACTH

Standard curve from a representative assay for adrenocorticotrophin (ACTH). Each point represents the mean of three replicates.

evaporation under vacuum, the label was dissolved in 1ml toluene/ethanol 9:1 and stored at -40°C.

2.5.3 Extraction of plasma

500cpm of ^{3}H-corticosterone in 100µl MeOH was added to each glass tube used for extraction and evaporated to dryness. 20µl plasma + 80µl distilled water for each sample was added, vortexed and left at room temperature for 10 min. Corticosterone was extracted by addition of 1ml ether, vortexed and left for 10 min at room temperature. The phases were separated by freezing in a solid CO_2 ethanol bath. The combined extracts were evaporated to dryness and reconstituted in 2ml methanol. 500µl was taken from each sample and counted for recovery of corticosterone. The percentage recovery was estimated per sample by multiplying recovery cpm by 4 and dividing by total counts. Samples were assayed in duplicate by aliquoting 2 x 100µl of the extract into LP3 tubes and evaporating to dryness.

2.5.4 Standards

A stock solution of corticosterone at 20.34mg/ml in ethanol, stored at -40°C was diluted immediately before use in methanol to give standards, in duplicate, of 20.3, 40.6, 81.2, 162.4, 324.8, 649.6 and 1299pg/tube. The standards were evaporated to dryness before inclusion in the assay.

2.5.5 Assay Procedure

The following procedure was used, all incubations were carried out at 4°C.

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Day 1 Samples and standards reconstituted in 100µl assay buffer and 100µl assay buffer containing 5,000cpm ^{3}H-Corticosterone was added, followed by 500µl assay buffer containing 1:5000 dilution of anti-corticosterone serum.*

Day 2 200µl assay buffer containing 0.5% (w/v) activated charcoal and 0.05% (w/v) Dextran T70 was added and left on ice for 20 min. The tubes were then centrifuged at 1,720 x g, 4°C for 20 min. Aliquots of 500µl were put into liquid scintillant and counted by a Packard liquid scintillation analyses.

A standard curve was calculated as described in 2.3.6.2 and a representative curve is shown in Fig. 2.9.

2.6 RADIOIMMUNOASSAY FOR VASOPRESSIN (AVP) AND OXYTOCIN (OT) (based on the method of Robinson, 1980)

2.6.1 Radioimmunoassay Buffer

The standard assay buffer used was 0.1M Tris containing 3% (w/v) BSA.

2.6.2 Iodination

The reagents used were:

Columns: For both VP and OT: A 20cm x 1cm Sephadex A-25 column, eluted with 0.1M Tris/3% BSA.

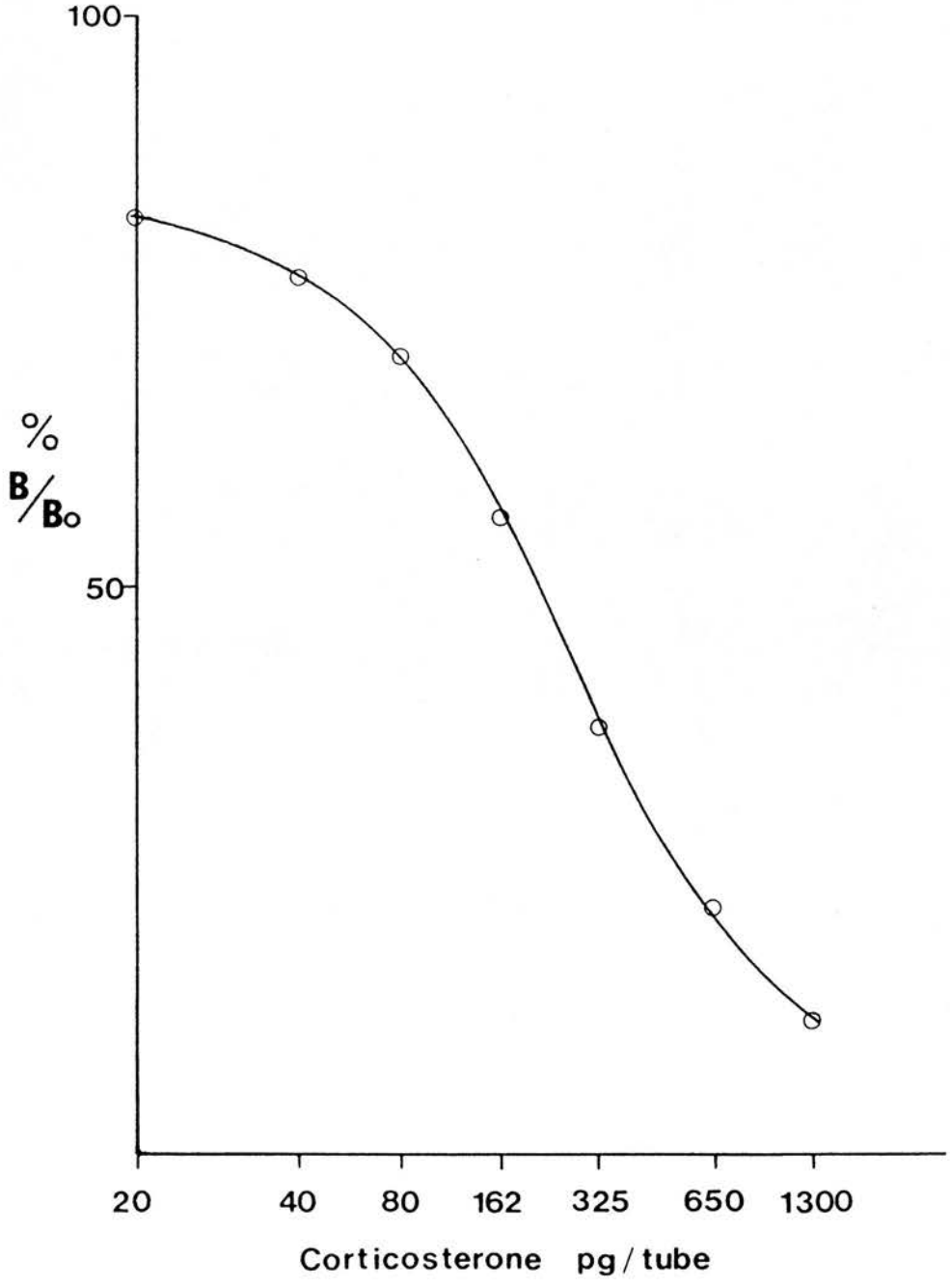


Figure 2.9 Standard curve for corticosterone

Standard curve from a representative assay for corticosterone. Each point represents the mean of two replicates.

Hormones: Oxytocin (440IU/mg; Dr. H. Vilhardt, Ferring, Sweden): 250µg ml^{-1} 0.2M phosphate buffer, 20µl aliquots were stored at -20°C until required. Vasopressin (400IU mg^{-1} Batch No. 770110; Ferring, Sweden): 250µg ml^{-1} 0.2M phosphate buffer, 20µl aliquots were stored at -20°C until required.

$Na^{125}I$: 1mCi in 10µl for each assay.

Iodogen: 2.5µg, deposited in a small glass tube.

Method: 5µg of the hormone was added together with $Na^{125}I$ to a tube containing Iodogen and incubated at room temperature for 20 min. The reaction mixture was transferred to a column and eluted with assay buffer and 1ml fractions were collected. The iodinated peptide appears as a single broad peak, this peak was rechromatographed on a 1 x 10cm sephadex A-25 column and the fractions tested for binding to excess antibody. The fraction showing best immunoreactivity was divided into 1µCi aliquots and stored at -20°C.

2.6.3 Standards

A standard curve for oxytocin was constructed using the IVth International Standard for OT (NIBS*) and for vasopressin using the 1st International standard for AVP (NIBS*). Standards were made up to 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0, 256.0 pg/tube immediately before use by doubling dilutions in 10% (v/v) cleared dog plasma in assay buffer from a stock solution of 5ng/ml stored at -20°C.

* NIBS - National Institute of Biological Standards

2.6.4 Assay Procedure for AVP

The following assay protocol was used, all incubations were carried out at 4°C.

Day 1 10µl samples and 90µl assay buffer or 100µl standards were added to LP3 tubes followed by 200µl assay buffer containing 3,000cpm ^{125}I-AVP and 1:5000 specific anti-AVP serum. The tubes were mixed and incubated overnight.

Day 2 900µl absolute alcohol was added to each tube and centrifuged at 3000 x g for 10 min at 4°C. The supernatant was aspirated and the pellet counted. Standard curve was calculated as described in 2.3.6.2 and a representative curve is shown in Fig. 2.10.

2.6.5 Assay Procedure for OT

The following assay protocol was used, all incubations were carried out at 4°C.

Day 1 10µl samples and 90µl assay buffer or 100µl standards were added to LP3 tubes followed by 200µl assay buffer containing 3,000cpm ^{125}I-OT and 1:225,000 specific anti-OT-serum. The tubes were mixed and incubated overnight.

Day 2 900µl absolute alcohol was added to each tube and centrifuged at 3,000 x g for 10 min at 4°C. The supernatant was aspirated and the pellet counted. A standard curve was calculated as described in 2.3.6.2 and a representative curve is shown in Fig. 2.11.

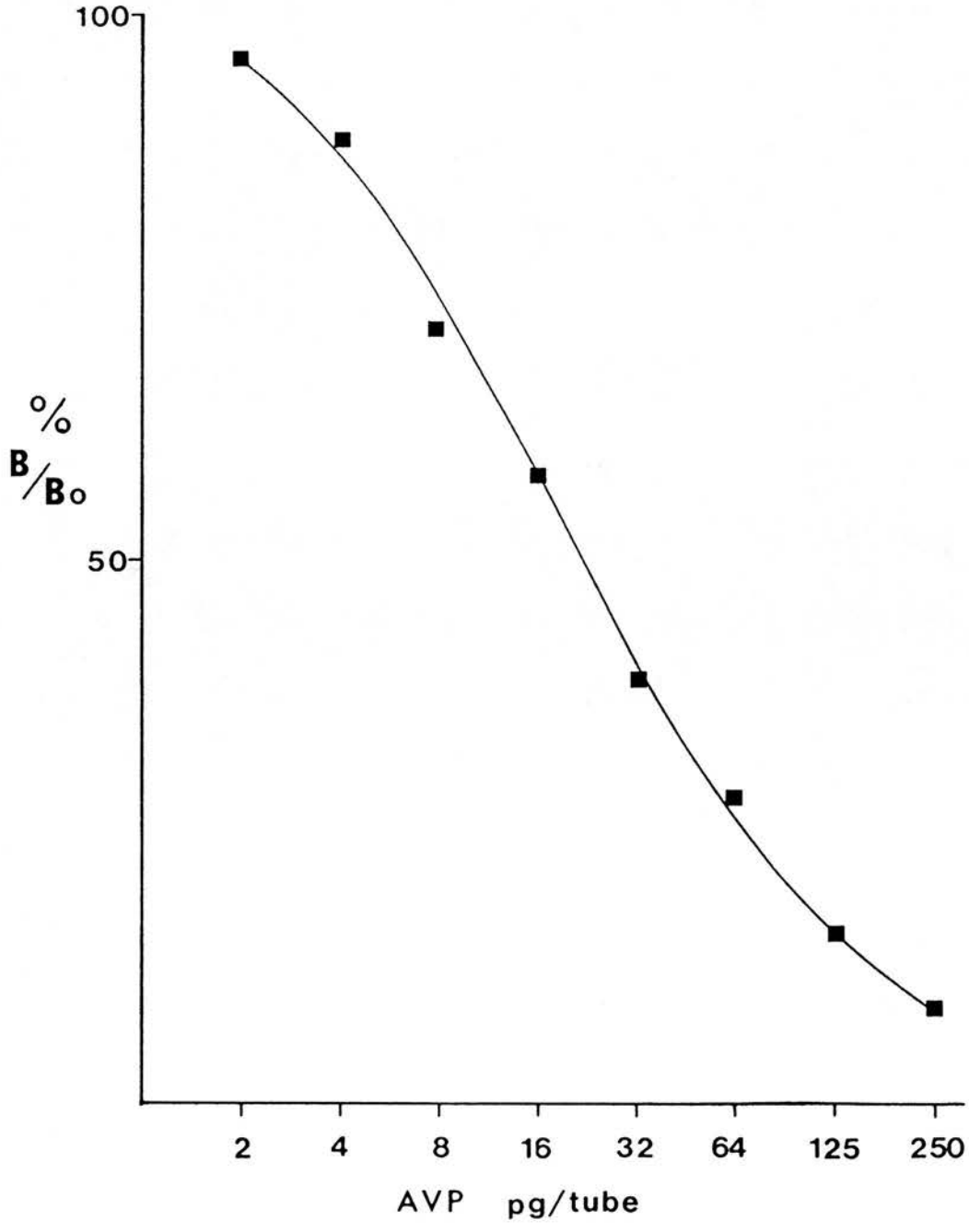


Figure 2.10 Standard curve for vasopressin

Standard curve from a representative assay for vasopressin (AVP) as described in section 2.6. Each point represents the mean of two replicates.

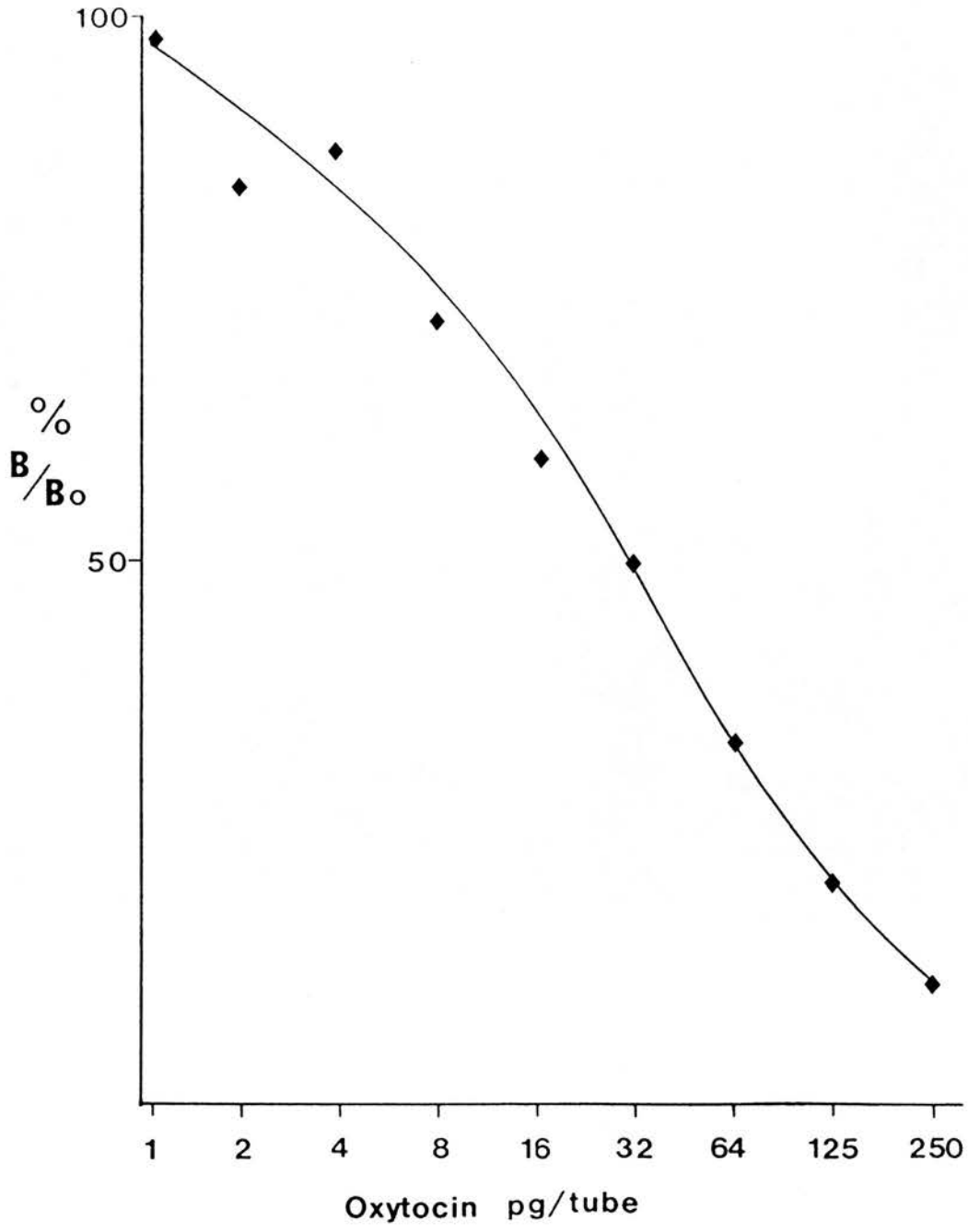


Figure 2.11 Standard curve for oxytocin

Standard curve from a representative assay for oxytocin. Each point represents the mean of two replicates.

2.7 RADIOIMMUNOASSAY FOR VASOPRESSIN

2.7.1 Radioimmunoassay Standard Diluent

RIA standard diluent consisted of RIA Buffer pH 7.4 + 0.1% Triton X-100 (see Appendix I)

2.7.2 Reference Standard

Standard curves were constructed using arginine vasopressin (Cambridge Research Biochemicals); 5, 10, 20, 50, 100, 200 and 500pg/ml were made up in RIA buffer containing 10% rat plasma and stored in 100µl aliquots at -40°C.

2.7.3 Antiserum

Rabbit anti-Arginine[8] vasopressin (RAS 8103) was obtained from Peninsula Labs Inc. and diluted 1:4 in RIA standard diluent from the stock provided by Peninsula. This dilution gave total binding (Bo) of approximately 25% of the total counts.

2.7.4 Labelled Antigen

Vasopressin was iodinated as described in 2.6.2 with the exception that iodinated vasopressin was separated from the crude reaction mix by HPLC as described in 2.3.3. Labelled vasopressin was stored at -70°C for up to three months.

2.7.5 Separation

Separation was by the double antibody technique. ARGG (SAPU) was diluted 1:10 in RIA buffer and NRS diluted 1:100 in RIA buffer. NRS and ARGG were added separately to each tube.

2.7.6 Assay Procedure

The following assay procedure was used, all incubations were at 4°C.

Day 1 100µl of standard or sample made up to 100µl in standard diluent was mixed with 100µl anti-AVP serum diluted in standard diluent.

Day 2 100µl of ^{125}I-AVP containing 10,000cpm in standard diluent was added and the tubes were mixed.

Day 3 350µl NRS 1:100 in RIA buffer and 350µl ARGG 1:10 in RIA buffer were added and the tubes mixed.

Day 4 1ml ice cold phosphate buffered saline was added before centrifuging at 4°C, 1,720 x g for 30 min. The supernatant was aspirated and the pellet counted. A standard curve was calculated as described in 2.3.6.2 and a representative curve is shown in Fig. 2.12.

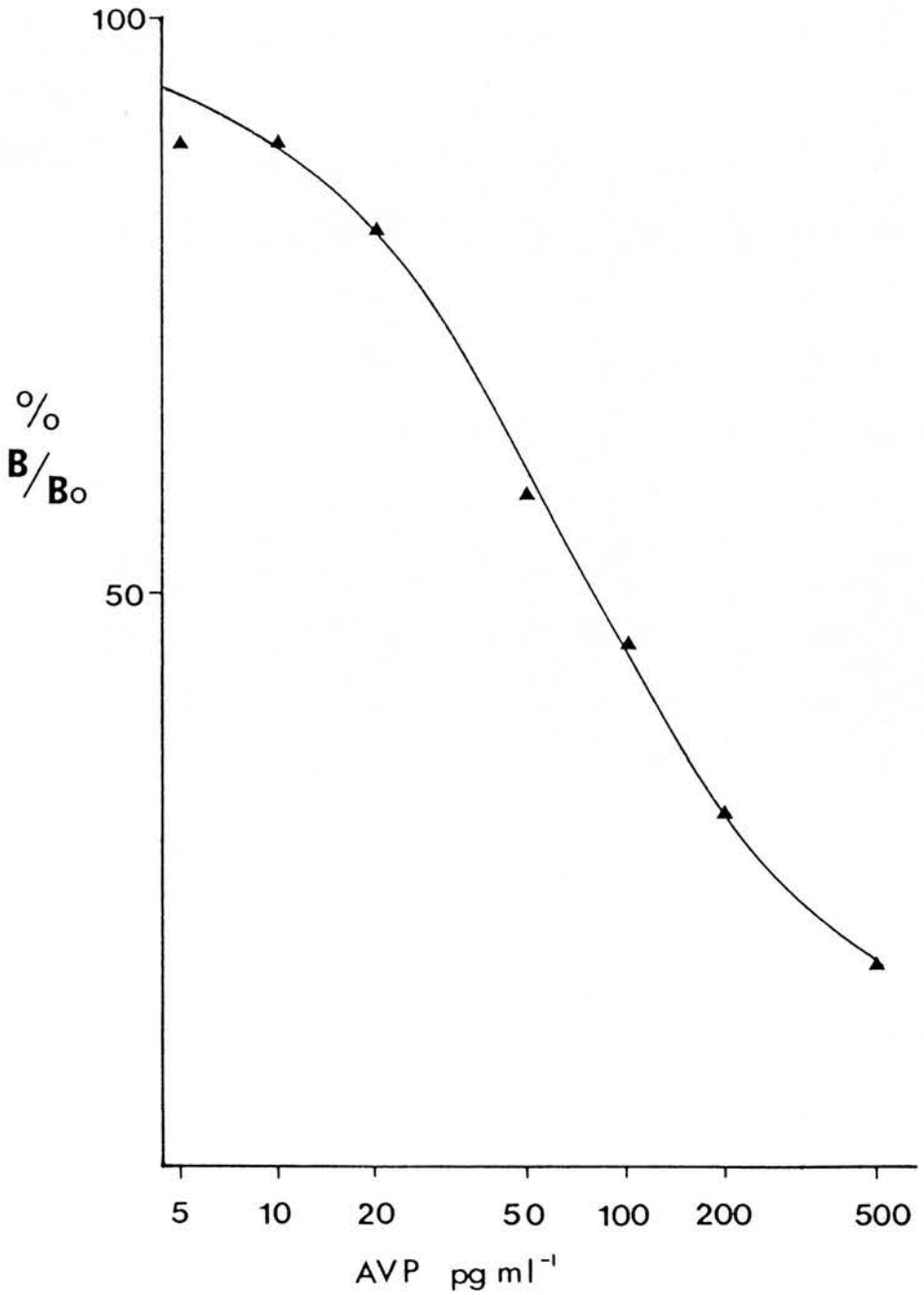


Figure 2.12 Standard curve for vasopressin

Standard curve from a representative assay for vasopressin as described in section 2.7. Each point represents the mean of three replicates.

CHAPTER 3

EFFECTS OF ENDOCRINE MANIPULATIONS ON THE CONTENT OF PROOPIOMELANOCORTIN mRNA IN THE ANTERIOR PITUITARY

3.1 INTRODUCTION

The gene encoding the protein pro-opiomelanocortin (POMC) in the mammalian neuroendocrine system is under complex hormonal control and is expressed in a tissue specific manner (Herbert et al., 1980; Vale et al., 1981; Rosa et al., 1980; Roberts et al., 1982). In the corticotrophic cells of the anterior pituitary, POMC is processed to form ACTH, β-lipotrophin and amino terminal peptide (Roberts et al., 1978; Hinman & Herbert, 1980). The POMC gene is also expressed in the neurointermediate lobe (NIL) of the pituitary gland and at low levels in the hypothalamus (Civelli et al., 1982; Roberts et al., 1982). However, different post-translational processing results in the secretion of different sets of hormones: ACTH in the NIL is further processed to α-MSH and CLIP (Eipper & Mains, 1980) and in the arcuate region of the hypothalamus, a mixture of ACTH, α-MSH and CLIP is produced (Krieger & Liotta, 1979).

Secretion of ACTH from the corticotroph cells of the anterior pituitary is stimulated by CRH (Vale et al., 1981; Yates & Maran, 1974; Rivier et al., 1982) and this action of CRH is potentiated by AVP (Vale et al., 1981; Gillies et al., 1982; Yasuda et al., 1982). Corticosteroids are secreted from the adrenal gland primarily in response to ACTH (Jones et al., 1976) and glucocorticoids produce inhibitory effects ('negative feedback') on the CRF activity of the hypothalamus (Buckingham, 1979) and on the secretion and content of pituitary ACTH (Dallman et al., 1972; Sayers & Portanova, 1974). In contrast, glucocorticoids have no influence on the secretion or content of POMC derived peptides in the NIL (Roberts et al., 1982). However, dopamine agonists have been shown to inhibit the release and content of α-MSH and

β-endorphin from the NIL (Vermes et al., 1980; Farah et al., 1982; Lepine & Dupont, 1981), while dopamine antagonists have a stimulatory effect (Hollt et al., 1982). Dopaminergic compounds have not been shown to affect POMC peptide secretion from the anterior pituitary (Rosa et al., 1980). While much is known about factors modulating POMC peptide secretion from the pituitary, less is known about the regulation of gene expression, although differential regulation in the different pituitary lobes is suggested by the alternate processing pathways. The introduction of recombinant DNA technology has resulted in reliable assays for the measurement of specific mRNA levels.* Measurement of POMC mRNA content by Herbert et al. (1981) in separated pituitary lobes after adrenalectomy showed progressively increasing content in the anterior pituitary after an initial lag period of 6h until they were 10-fold higher than control values; no change in POMC mRNA content was found in the NIL. Dexamethasone treatment decreased POMC mRNA content 30- to 50-fold in adrenalectomised animals. Administration of glucocorticoids produces a dramatic reduction in POMC gene transcription which can be detected 15 min after the injection. This shows that the inhibitary actions of glucocorticoids on POMC mRNA are due, at least in part, to an inhibition in POMC mRNA synthesis (Eberwine & Roberts, 1984).

The importance of normal PVN function on the response of corticotrophs to adrenalectomy has been suggested by results which showed that the high levels of POMC mRNA in corticotrophs after adrenalectomy decrease after the PVN is lesioned (Bruhn et al., 1984). The increase in POMC synthesis after adrenalectomy may be accounted for largely by the removal of corticosteroids. However,

* For details of 'Northern' blotting see Appendix II.

in the absence of hypothalamic releasing factors due to hypothalamic deafferentation, no increase in POMC mRNA after adrenalectomy is observed (Dallman et al., 1985).

Long-term exposure of cultured adenohypophysial cells to ovine-CRH increases the cellular content and secretion of ACTH (Vale et al., 1983b) and also increases the content of POMC mRNA in corticotrophs (Bruhn et al., 1984). Long-term administration of ovine CRH *in vivo* increased POMC mRNA content in the anterior pituitary gland and this increase was reversed by injection of dexamethasone (Bruhn et al., 1984). Little is known of the effect, if any, of AVP on ACTH synthesis.

The aims of the present study were to measure POMC mRNA content in the anterior pituitary gland and examine the effects on POMC gene expression (i) of high and low concentrations of glucocorticoids in plasma and (ii) increased CRH release into hypophysial blood by electrical stimulation of the hypothalamus, and (iii) to determine the importance of normal vasopressin levels in the brain on POMC gene expression. Conditions of low plasma glucocorticoid concentration were produced by adrenalectomy and high plasma glucocorticoid concentrations were produced by ether stress. It is well established that a variety of stressors cause release of ACTH from the anterior pituitary gland and glucocorticoids and catecholamines from the adrenal gland, but there are few data on the effects of stress on the biosynthesis of POMC. In order to investigate whether POMC gene expression can be increased by an increased release of endogenous CRH into portal blood, anterior pituitary contents of POMC mRNA after electrical stimulation of the PVN were compared with those after electrical stimulation of the

amygdala. The PVN were stimulated because they contain the highest concentration of CRH and because stimulation of the PVN results in increased CRH release in portal blood (see Chapter 7). Control animals with electrode implants in the amygdala, were used to determine whether changes, if any, in POMC gene expression were due simply to the stress of implantation of electrodes. The dependance of POMC gene transcription and translation on normal hypothalamic AVP levels was determined by measuring the content of POMC mRNA in the anterior pituitary gland in Brattleboro rats (as defined in section 2.1) which are genetically deficient in AVP due to a single base deletion of the gene (Schmale & Richter, 1984). Lack of AVP expressed in magnocellular neurones results in diabetes insipidus and Brattleboro rats are continuously searching for water. The effect of replacing AVP in the Brattleboro rat with the synthetic analogue, dDAVP, on POMC mRNA content in the anterior pituitary gland was also examined.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Adult female Wistar COB rats (200-250g body weight) purchased from Charles River U.K. Ltd. (Margate, Kent) were used for adrenalectomy, control and stress experiments. Homozygous or heterozygous Brattleboro rats (200-250g body weight) were bred in the Department of Pharmacology. Adult female Long Evans rats were used as controls and were purchased from Charles River U.K. Ltd. The animals were maintained under controlled lighting (lights on 0500-1900h) and temperature (22°C) and had free access to Diet 41B (Oxoid, Basingstoke, Hants) and tap water. Animals that had been

operated on drank 0.9% (w/v) NaCl solution containing 40mg/l chlortetracycline hydrochloride.

Brattleboro rats were caged singly in metabolic cages to measure urine output. A homozygous Brattleboro rat was defined as an animal which passed urine equal to or greater than 75% of body weight during a 24h collection period. Brattleboro rats passing less than this were classed as heterozygous.

Ether stress was applied to female Wister COB rats by placing them in a chamber permeated with ether vapour for 5 min until the animals were unconscious. Trunk blood was collected from rats killed without ether stress (cont), immediately after (0h), and 6h and 24h after the stress. There were five animals in each of the four groups. The animals were rapidly decapitated and the blood was collected in chilled polypropylene tubes washed with 1000U heparin in 0.9% saline. Trasylol, 1000 KIU/ml blood was added immediately and samples mixed. Plasma was stored at -40°C.

3.2.2 RNA Extraction

The pituitary gland was quickly removed and the anterior and neurointermediate lobes were separated using watchmaker forceps. RNA was extracted according to Chirgwin et al. (1979). Freshly dissected tissues were immediately homogenised in 10 volumes of 4M guanidinium thiocynate and 1M mercaptoethanol pH 5.0 and left at room temperature for 2h. The homogenate was loaded onto a 5.7M ceasium chloride cushion and centrifuged at 138,900 x g for 20h at room temperature. The resultant pellet was dissolved in 100mM Tris acetate/100mM sodium acetate pH 9.0 and the RNA precipitated with 10% (v/v) 2M potassium acetate pH 5.5 and 10 volumes ethanol. The

amount of RNA in the pellet was determined by dissolving the pellet in water and measuring uv absorbance at 260nm. RNA was stored at 2.5μg/μl at -70°C.

3.2.3 Isolation and in vitro translation of mRNA

Poly (A^+) RNA was prepared from total RNA by chromatography on an oligo (dT) cellulose column as described by Aviv & Leder (1972). RNA was bound to the oligo (dT) cellulose in high salt binding buffer (0.5M NaCl, 1mM EDTA, 10mM Tris pH 7.5) and eluted from the column with low salt buffer (1mM EDTA, 10mM Tris pH 7.5). 0.7ml fractions were collected and those containing Poly (A^+) RNA, as shown by uv absorbance at 260nm, were pooled and precipitated. Poly (A^+) RNA was stored at 1μg/μl at -70°C.

The activity of Poly (A^+) RNA isolated in this manner was checked by inclusion into a cell free rabbit reticulocyte-lysate. Isolated Poly (A^+) RNA added to the reticulocyte lysate system increased incorporation of ^{35}S-methionine into precipitating proteins 10-fold over control values. Thus the mRNA was judged to be intact and capable of being translated.

3.2.4 Isolation of Recombinant Plasmid DNA

A 550 base pair cDNA complementary to the ACTH and β-LPH sequence of the rat POMC mRNA was inserted into pBR322 at the Pst 1 site (Drouin & Goodman, 1980). Bacterial growth and plasmid preparation were as previously described (Clewell & Helinski, 1972). Cells were centrifuged, lysed and the plasmid concentrated by centrifugation in ceasium chloride. The plasmid was then purified by gel filtration chromatography on a Sephadex G50 column

equilibrated in Buffer H (10mM Tris, 100mM NaCl, 1mM EDTA, 0.1% (w/v) SDS pH 7.6).

For Northern Blot analysis the unrestricted plasmid was labelled with [α^{32}P] dCTP by nick translation (Amersham Nick Translation Kit N150) to a specific activity of 10^8 cpm/µg DNA. The labelled plasmid was separated from free [α^{32}P]-dCTP by precipitation with 2.5M ammonium acetate using tRNA as a carrier.

3.2.5 Preparation of Markers

Plasmids pBR322 and pAT153 were restricted with enzymes Alu and Pst 1 and Bam H1 respectively and heat inactivated at 65°C for 10 min. The reactions were mixed giving restriction fragments of 2525, 1131, 910, 657/655, 521, 403 and 281 base pairs. The POMC cDNA probe is inserted into pBR322 and, therefore, the nick translated plasmid will hybridise with markers derived from pBR322. Markers were run with each set of samples.

3.2.6 Detection of POMC mRNA

3.2.6.1 Preparation of RNA sample for Nothern Blot

The RNA was dissolved in 4.4µl H_2O and 2.0µl 10 x MEA (running buffer) was added to give a final concentration of 1 x MEA. The sample was then denatured by the addition of 3.6µl formaldehyde and 10.0µl formamide and further denatured by heating at 60°C for 15 min. Samples and markers were treated the same way.

3.2.6.2 RNA Separation

RNA samples and markers were separated by electrophoresis on a

1.1% agarose gel containing 6.7% Formaldehyde and 1 x MEA buffer. Bromophenol blue was added to the markers as a front indicator. The gel was run at 150V, 60-80mA, in 1 x MEA until the front was 1cm from the end of the gel.

3.2.6.3 Hybridisation of Probe

The RNA from the gel was transferred on to presoaked nitrocellulose sheets according to Thomas (1980). The blots were then baked at 80°C for 4 hrs and prehybridised in a sealed plastic bag for 4 hrs at 42°C containing 20mls prehybridisation buffer (50mM Tris-HCl pH 7.6, 1M NaCl, 0.1% tetrasodium pyrophosphate, 50µg ml^{-1} denatured single strand salmon sperm DNA, 10 x denhardt's reagent, 50% formamide). The buffer was then replaced with 10ml prehybridisation buffer containing 5% (w/v) dextran sulphate (Sigma Chemicals) and nick translated rat POMC cDNA (section 3.2.4) after heat denaturing for 5 min. Hybridisation was carried out at 42°C in a shaking water bath for 16-20 hrs. Excess probe was removed from the blot with repeated washing with 5mM Tris HCl pH 7.5, 25mM NaCl, 0.1% SDS, 1mM EDTA at 52°C for 2h. The nitrocellulose was dried and autoradiographed with the aid of intensifier screens.

3.2.7 Quantitation of POMC mRNA

Amounts of RNA loaded onto gels and then subsequently transferred onto nitrocellulose are not always consistent and, therefore, an internal control was used to standardise the samples. Each blot was probed with POMC cDNA and then the procedure repeated with mouse 7SL cDNA as the probe (Balmain et al. 1982).* In some cases, both probes were added together. The

proportion of 7SL RNA in a given cell population will be constant and, therefore, changes in POMC mRNA levels can be related to it. Autoradiographs were obtained for POMC and 7SL and the intensity of the bands determined quantitatively by scanning densitometry and integration of the peak. Results are expressed as a ratio of POMC mRNA/7SL RNA.

3.3 RESULTS

3.3.1 Detection of POMC mRNA in total and Poly (A^+) RNA extracts

Amounts of Poly (A^+) and total RNA extracts from the anterior pituitary gland of female Wistar COB rats (200-250g body weight) were size-fractionated on an agarose gel. The resulting Northern blot was then hybridised with the specific POMC cDNA probe and with a specific 7SL cDNA probe. Figure 3.1 shows a very strong signal from the Poly (A^+) enriched RNA extract corresponding to a size of approximately 1000 bases and a weaker one from the total RNA. As expected only total RNA tracks gave a signal for 7SL RNA. Figure 3.1 shows that POMC mRNA can be detected in total RNA extracts and also that 7SL RNA content can be used as an internal standard. Amounts of RNA loaded onto the gel were routinely within 10% of each other.

3.3.2 Glucocorticoid Effect on POMC mRNA Levels

Total RNA was extracted from anterior pituitaries of adult female Wister COBS and from female Wistar COBS that had been adrenalectomised three weeks previously. Adrenalectomy was validated by the presence of very low plasma corticosterone concentrations and by inspection for possible adrenal remnants at

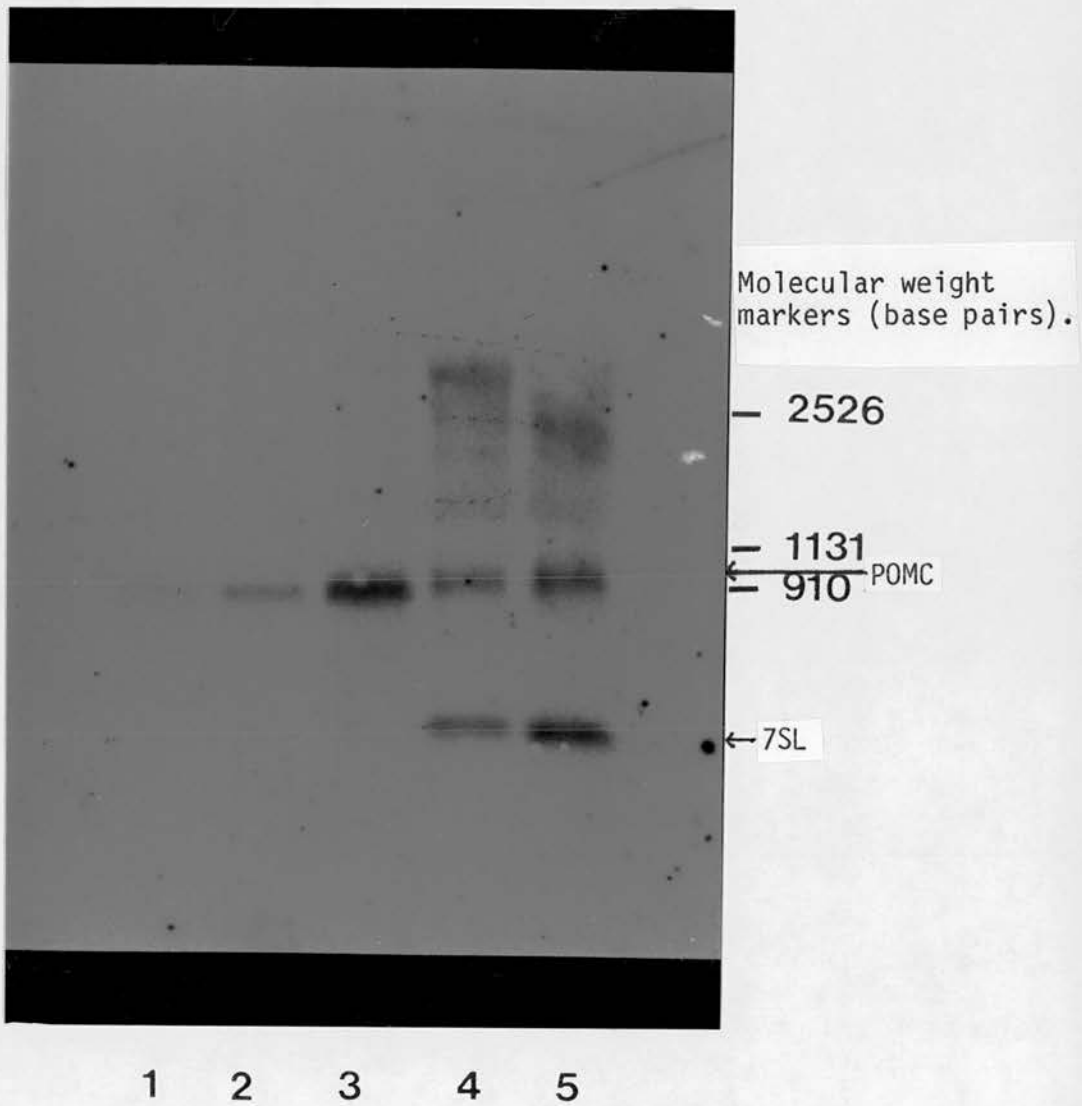


Figure 3.1 Detection of POMC mRNA in total and Poly (A^+) RNA extracts from the anterior pituitary gland

Northern blot analysis of Poly (A^+) RNA (lanes 1-3) and total RNA (lanes 4-5) isolated from the rat anterior pituitary gland hybridized with 7SL cDNA and POMC cDNA Probes. The amount applied was 0.5µg (lane 1), 1.0µg (lane 2), 2.0µg (lane 3), 5µg (lane 4) and 10µg (lane 5).

autopsy. POMC mRNA levels were determined as described and quantitated with respect to 7SL RNA levels. Figures 3.2 and 3.3 show that adrenalectomy increased the content of POMC mRNA in the anterior pituitary gland 10-fold over the control values.

3.3.3 Effect of Stress on POMC mRNA Levels

The three groups of five animals were stressed with ether as described in section 3.2.1 and decapitated 0, 6 and 24h after the stress together with a control, non-stressed group. Total RNA was extracted from the anterior pituitary glands of these animals and trunk blood was collected and assayed for ACTH and corticosterone. Figure 3.4 shows that immediately after stress, plasma ACTH concentrations had increased approximately 10-fold over control animals with a 3-fold increase in corticosterone. Six hours after stress, plasma ACTH concentrations had dropped, but were still 5-fold greater than control values. Twenty-four hours after the stress, the plasma ACTH concentrations were similar to those in control animals. POMC mRNA levels were determined in the three groups as described and were quantitated with respect to 7SL RNA levels. Figures 3.5 and 3.6 show POMC mRNA content in the anterior pituitary gland had increased 2-fold 6h after stress and had returned to control values 24h after the stress.

3.3.4 Effect of Electrical Stimulation on POMC mRNA Levels

Electrodes were implanted as described (2.2.8) and seven days later, the animals were anaesthetised with sagatal and stimulated for 30 min as described in 2.2.7. A control group of animals with electrode implants in the amygdala were anaesthetised but no

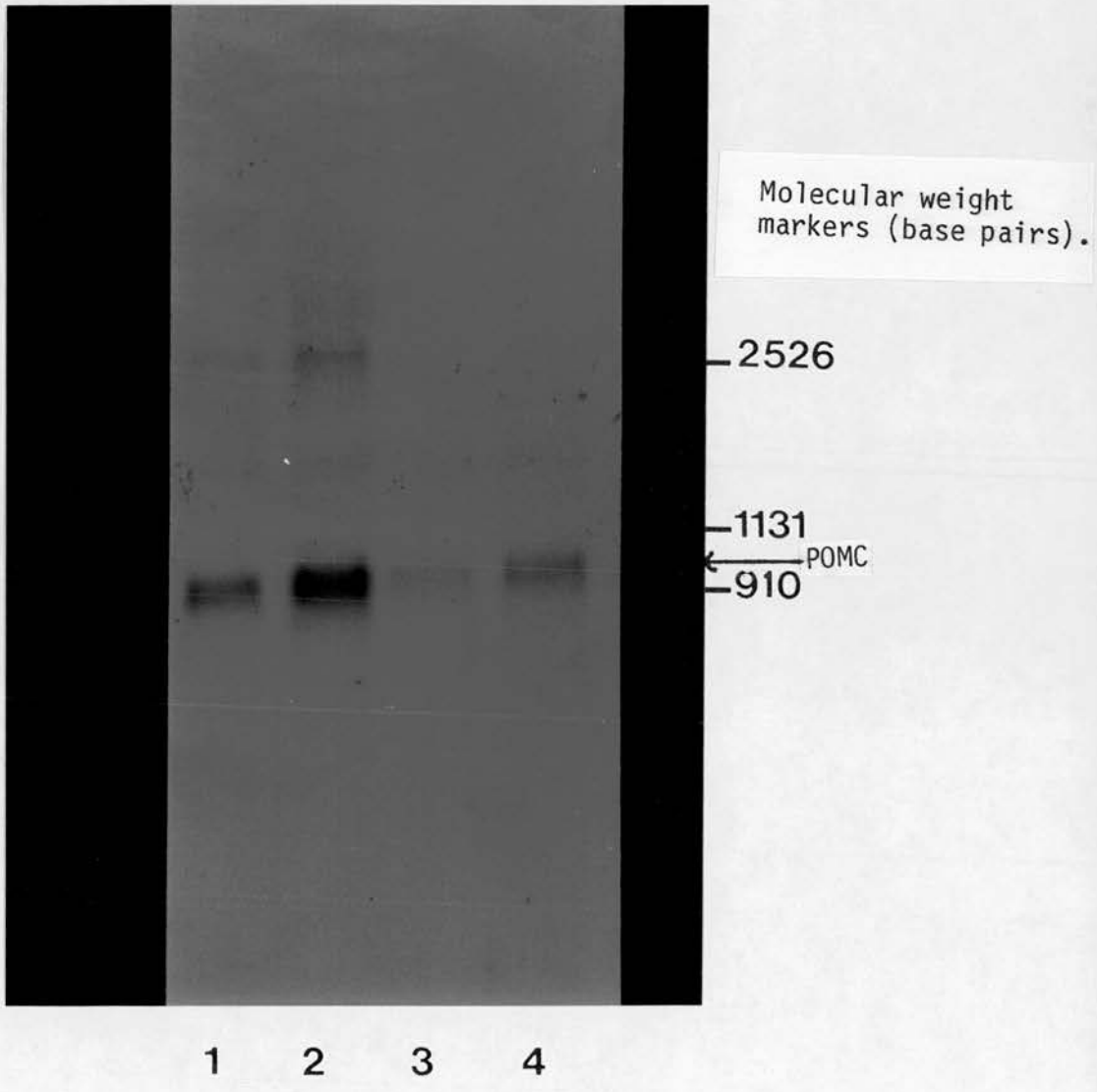


Figure 3.2 POMC mRNA content in the anterior pituitary gland of adrenalectomized rats

Northern blot analysis of total RNA isolated from the rat anterior pituitary gland from control female Wistar rats (lanes 3 and 4) and in female Wistar rats that had been adrenalectomised for three weeks (lanes 1 & 2) hybridised with a POMC cDNA probe. The amount applied was 5µg (lane 1), 10µg (lane 2), 5µg (lane 3) and 10µg (lane 4).

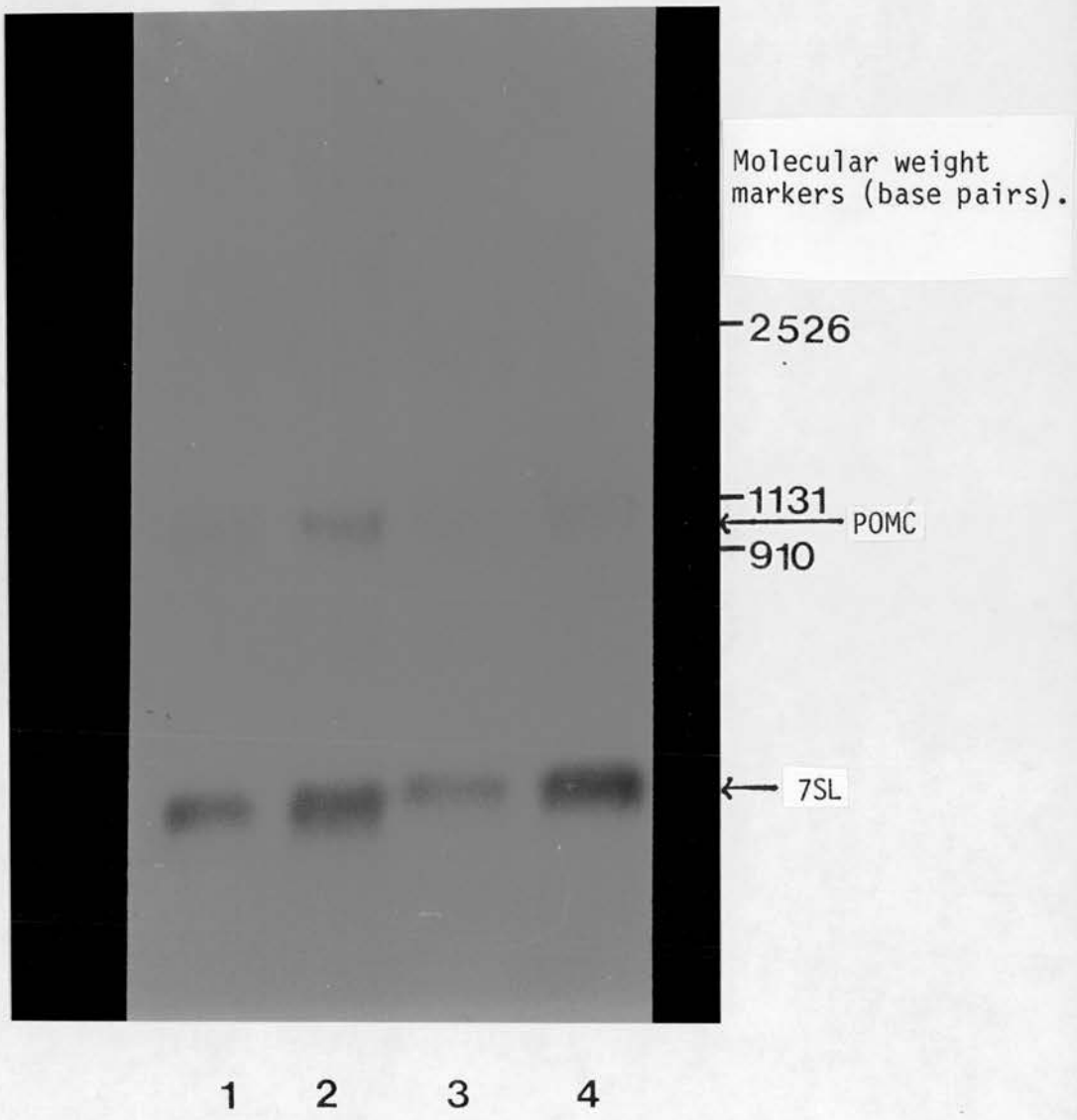
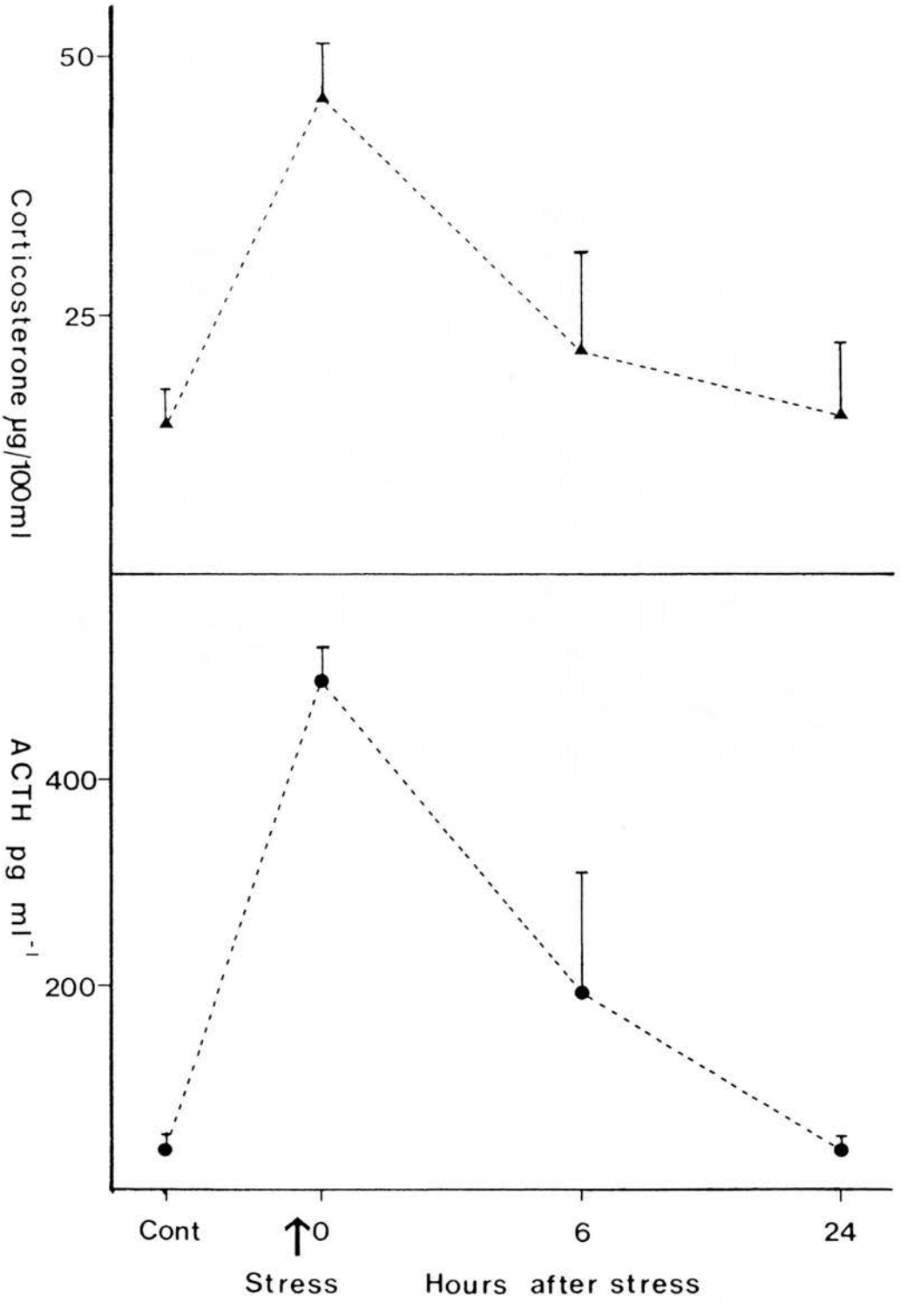


Figure 3.3 7SL RNA content in the anterior pituitary gland of adrenalectomised animals

Northern blot analysis of total RNA isolated from the rat anterior pituitary gland from control female Wistar rats (lanes 3 and 4) and in female Wistar rats that had been adrenalectomised for three weeks (lanes 1 & 2) hybridised with a 7SL cDNA probe. The amount applied was 5µg (lane 1), 10µg (lane 2), 5µg (lane 3) and 10µg (lane 4).

Figure 3.4 Effect of ether stress on ACTH and corticosterone concentrations

Mean (± S.E.M.; n = 6) concentrations of ACTH (pg ml^{-1}) and corticosterone (μg/100ml) in peripheral plasma from intact female Wistar rats before and after a 5 min period of ether stress. Trunk blood samples were taken immediately after decapitation before stress (Cont), immediately after stress (time 0) and 6h and 24h after stress.


50
25
Corticosterone µg/100ml
400
200
ACTH pg ml⁻¹
Cont
0
6
24
Stress
Hours after stress

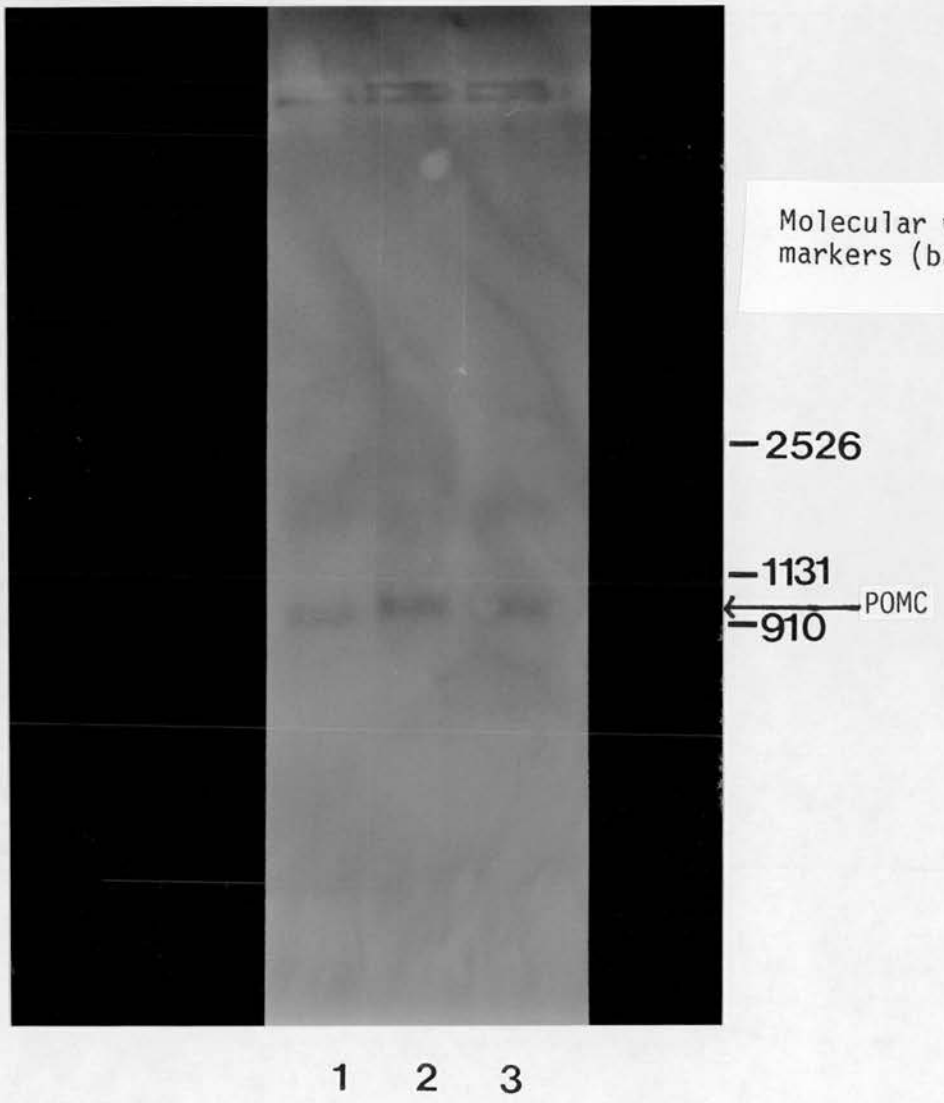


Figure 3.5 The effect of ether stress on POMC mRNA content in the anterior pituitary gland

Northern blot analysis of 10μg total RNA extracted from the anterior pituitary gland of female Wistar rats immediately after 5 min ether stress (lane 1), 6h after ether stress (lane 2) and 24h after stress (lane 3) hybridised with a POMC cDNA probe.

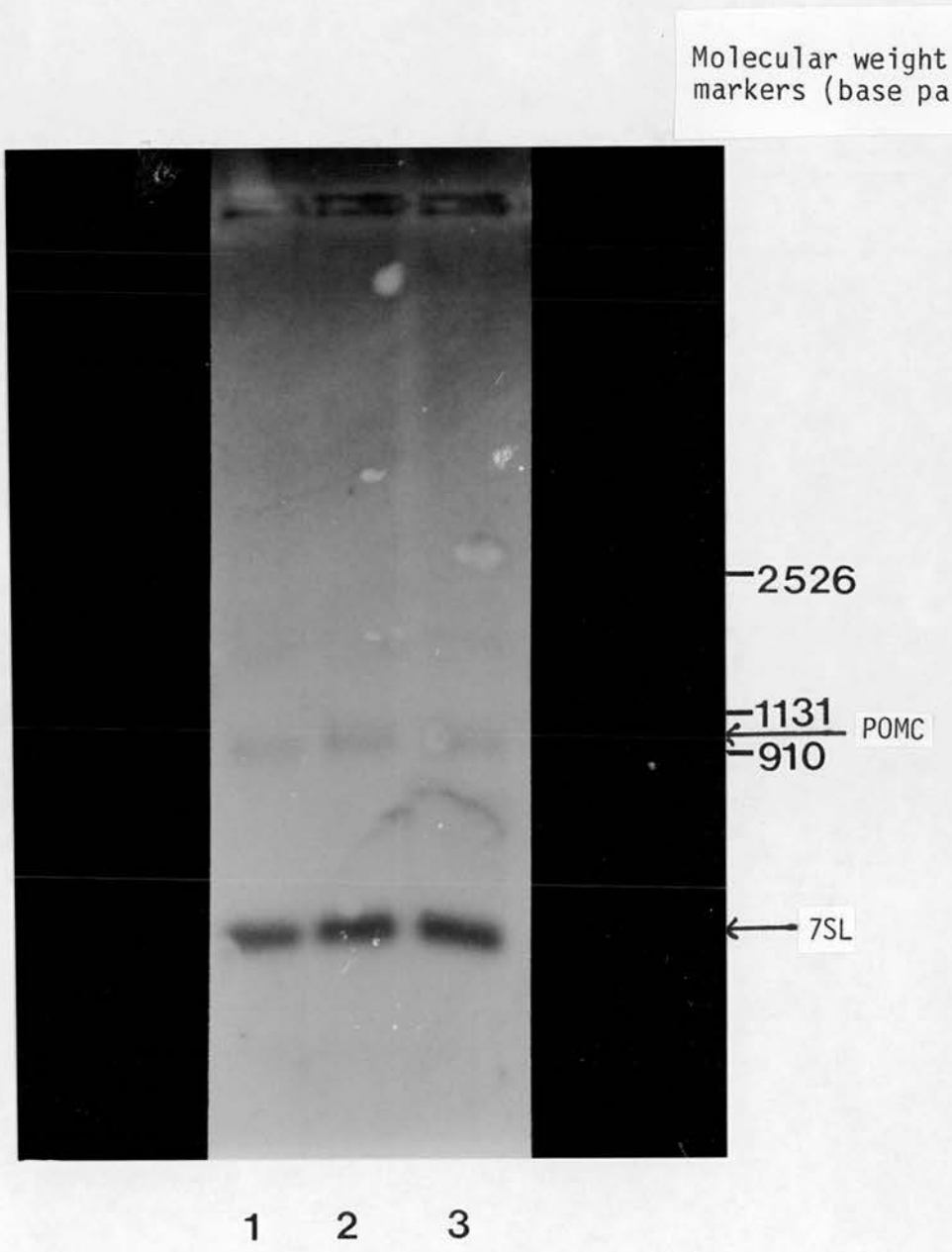


Figure 3.6 Content of 7SL RNA In the anterior pituitary gland of ether stressed rats

Northern blot analysis of 10µg total RNA extracted from the anterior pituitary gland of female Wistar rats immediately after 5 min ether stress (lane 1), 6h after ether stress (lane 2) and 24h after stress (lane 3) hybridised with a 7SL RNA probe.

stimulus was applied. Six hours after stimulation, the animals were decapitated and the anterior pituitary glands removed. Pituitary POMC mRNA contents were determined. Figure 3.7 shows that there was no detectable difference in POMC mRNA content between the stimulated and the non-stimulated animals.

3.3.5 POMC mRNA Levels in the Brattleboro Rat

RNA was extracted from the anterior pituitary of female Long Evans rats and female homozygous and heterozygous Brattleboro rats (as defined in 2.1) and POMC mRNA levels determined. Fig. 3.8 shows that the pituitary content of POMC mRNA in both homozygous and heterozygous Brattleboro rats was 2-fold higher than the content in the anterior pituitary glands from the Long Evans rats.

In order to establish whether this increase in POMC mRNA content in Brattleboro rats could be reversed by treatment with a AVP analogue, groups of heterozygous and homozygous Brattleboro rats were given 10μg/100g body weight dDAVP (Ferring) i.p. 24h and 1h before decapitation. Control Long Evans and Brattleboro rats were also decapitated at the same time after injection with 1ml/100g body weight 0.9% saline. RNA was extracted from the anterior pituitary glands and POMC mRNA content determined. Figure 3.9 shows that no difference in POMC mRNA content was detectable between the treated and non-treated Brattleboro rats.

3.4 DISCUSSION

These results confirm that POMC mRNA can be detected in total RNA extracts from the anterior lobe of the pituitary by Northern Blot analysis. The content of specific POMC mRNA has been

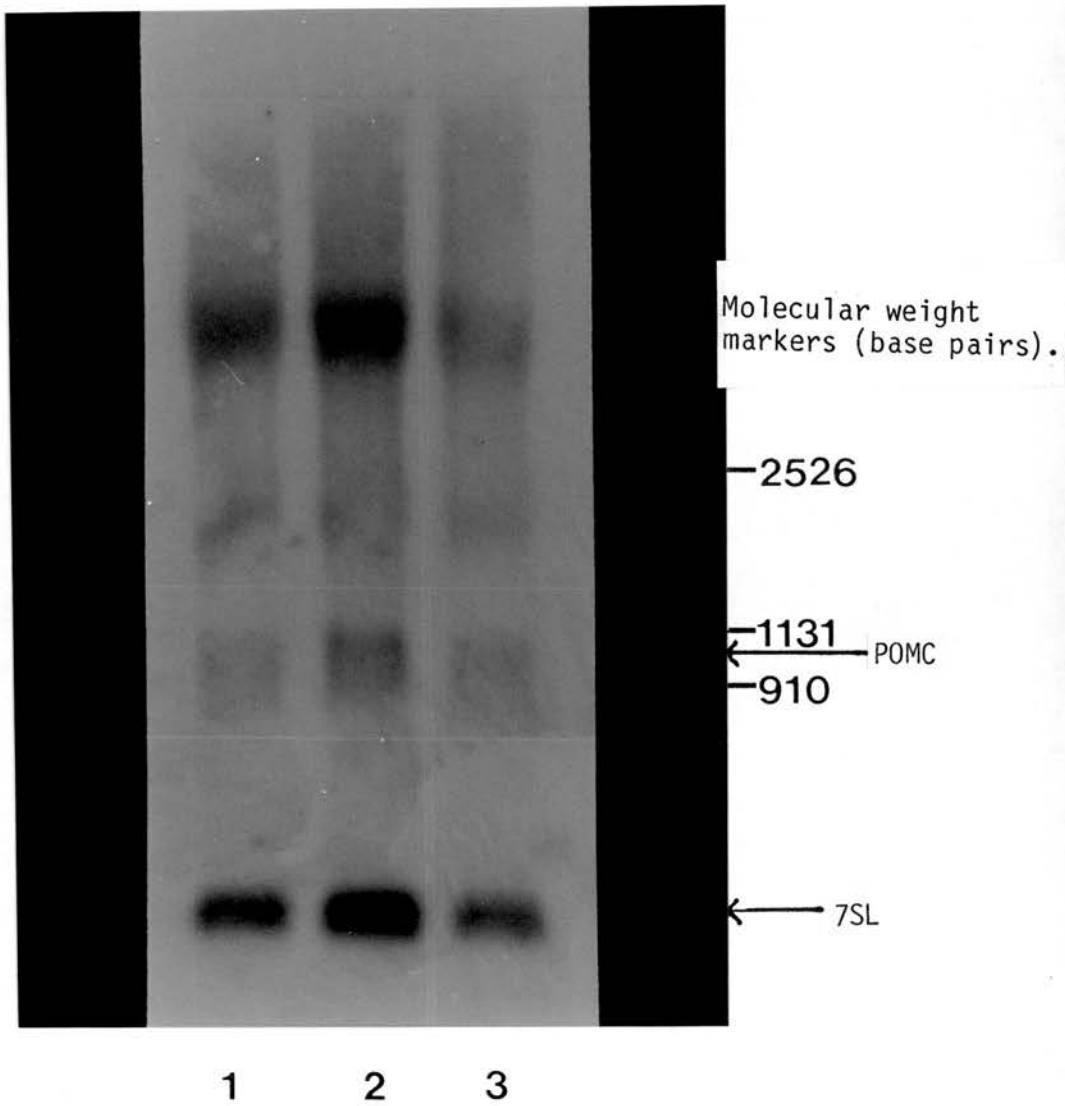


Figure 3.7 Effect of electrical stimulation of the brain on POMC mRNA content in the anterior pituitary gland

Northern blot analysis of 10µg total RNA extracted from the anterior pituitary gland of female Wistar rats 6h after electrical stimulation, hybridised with 7SL cDNA and POMC cDNA probes. In control animals (lane 1) electrodes were implanted in the amygdala but no stimulation was applied, electrical stimulation was applied to the amygdala (lane 2) and the PVN (lane 3) for 30 min (30s on, 30s off of biphasic rectangular pulses, 50Hz, 1mA peak to peak, 1ms duration).

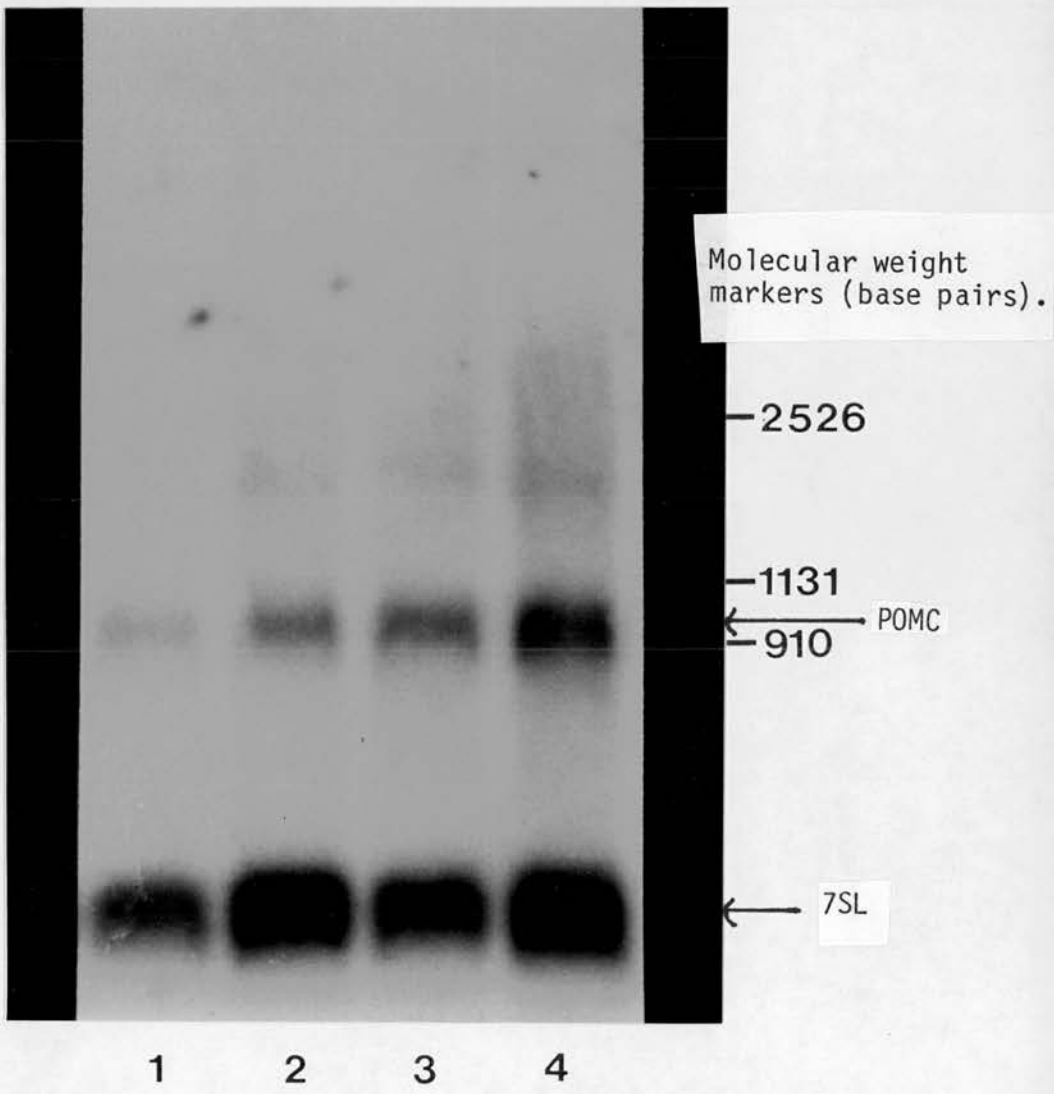


Figure 3.8 POMC mRNA content in the anterior pituitary gland of Brattleboro rats

Northern blot analysis of total RNA extracted from the anterior pituitary gland of Long Evans rats (lanes 1 & 2), heterozygous (lane 3) and homozygous (lane 4) Brattleboro rats hybridized with POMC cDNA and 7SL cDNA probes. The amounts applied were 5µg (lane 1), 10µg (lane 2), 5µg (lane 3) and 10µg (lane 4).

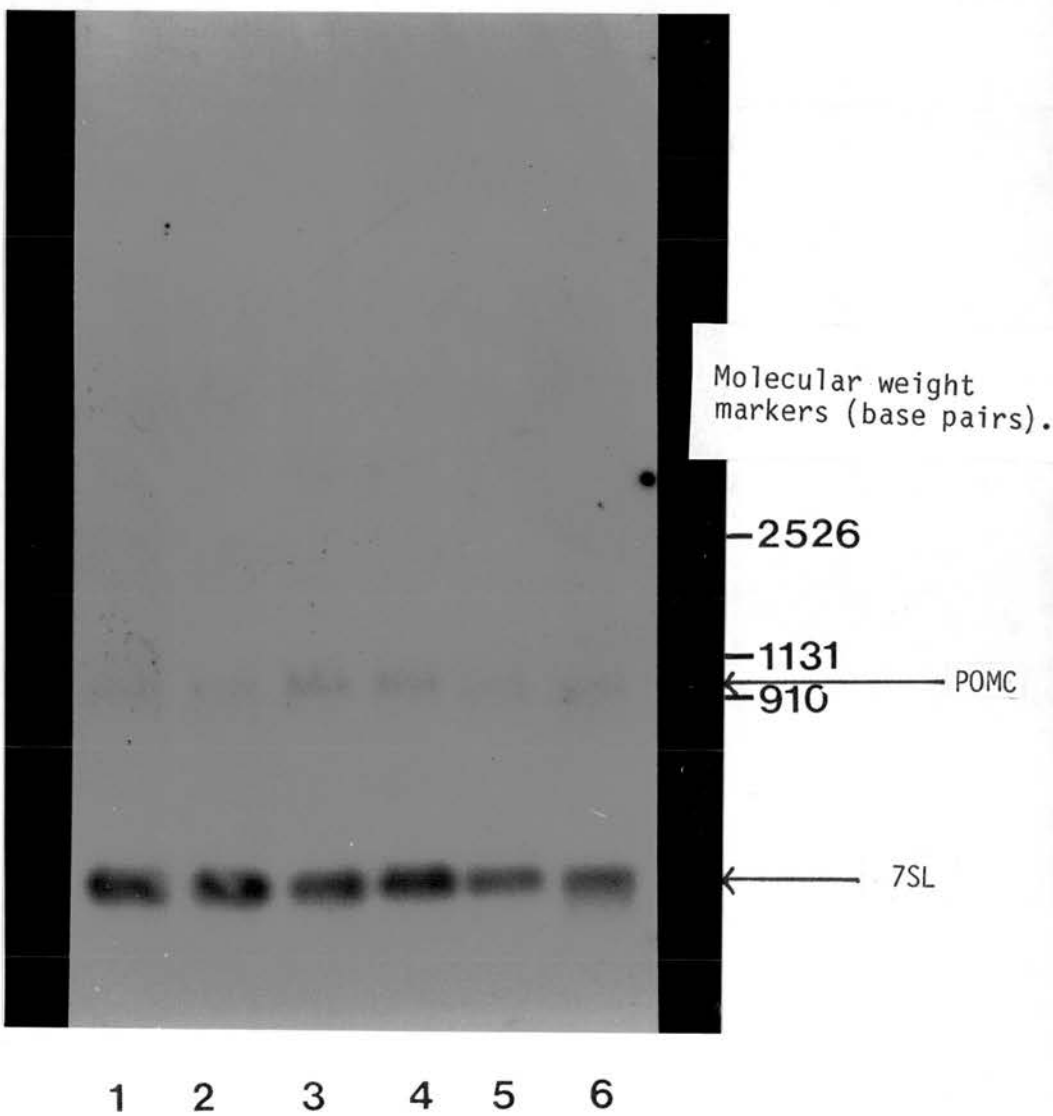


Figure 3.9 The effect of dDAVP on POMC mRNA content in the anterior pituitary gland of Brattleboro rats

Northern blot analysis of 10µg total RNA extracted from the anterior pituitary gland of Long Evans rats (lanes 1 & 2), heterozygous Brattleboro rats (lane 3), heterozygous Brattleboro rats injected with dDAVP (10µg/100g body weight) 24h and 1h before decapitation (lane 4), homozygous Brattleboro rats (lane 5) and homozygous Brattleboro rats injected with dDAVP as above (lane 6). The Northern blot was hybridised with POMC cDNA and 7SL cDNA probes.

quantitated with respect to content of 7SL RNA. POMC mRNA detected in this way has a mobility corresponding to an RNA of approximately 1000 bases which is in agreement with Civelli et al. (1981). Some higher molecular weight bands were detected and presumably correspond to larger, immature forms of the mRNA. Identification of specific POMC mRNA was further confirmed by the large increase in mRNA levels measured after bilateral adrenalectomy: three weeks after adrenalectomy, a 10-fold increase was found which corresponds well with other reports (Herbert et al., 1980; Bruhn et al., 1984). The increase in steady state mRNA content has been shown to be due to an increase in POMC gene expression 1h after adrenalectomy which can be reversed by administration of dexamethasone (Birnberg et al., 1983; Eberwine & Roberts, 1983). mRNA content continued to rise up till day 18 after adrenalectomy although the increase in transcription rates had reached a plateau by 14 days. This continued increase in mRNA content is unusual and is probably due to the complex effect of adrenalectomy on corticotroph cells. Five days after adrenalectomy, POMC-containing cells in the anterior lobe of the pituitary gland increased in size and number (Childs et al., 1982). In situ hybridisation has shown that after adrenalectomy in the rat there is a 2-fold increase in POMC mRNA density in corticotroph cells and a 3-fold increase in the number of cells in the anterior pituitary that express POMC mRNA (Gee et al., 1983). The plateau reached in the increase in transcription rates of the POMC gene by day 14 is likely due to the cell mass not increasing any further. The increase in POMC gene expression after removal of glucocorticoid by adrenalectomy suggests that in the intact animal, POMC gene expression is partially repressed by the presence of

endogenous glucocorticoids. However, Dallman et al. (1985) have shown that in the absence of endogenous drive from the hypothalamus, the corticotroph does not have the capacity to respond to adrenalectomy and that in the absence of drive from external sources, the corticotroph has no autonomy.

The results of the present studies suggest that three conclusions may be drawn about the regulation of the adrenocortical system: (1) acute stress which causes a discharge of ACTH from the pituitary gland is accompanied by an increase in POMC mRNA content in the anterior pituitary gland (2) stimulation of the PVN which leads to a short-term increase in exposure of the corticotrophs to CRH does not appear to affect the synthesis of ACTH in the anterior pituitary gland (3) lack of AVP in the Brattleboro rat is associated with results in an increased content of POMC mRNA in the anterior pituitary gland which is not affected by treatment with dDAVP.

Acute stress in the rat, elevated the levels of POMC mRNA in the anterior pituitary and this was associated with an increase in the plasma concentration of the POMC derived peptide, ACTH, and with an increase in the release of corticosterone from the adrenal gland. These results show that both the biosynthetic and secretory activities of the anterior pituitary are enhanced 6h after ether stress. Hollt et al. (1986) have reported an increase in POMC mRNA content in the anterior pituitary gland after footshock applied twice daily for up to seven days; the increase was already established by day one and diminished by day seven. The magnitude of the increase in POMC mRNA content seen after repeated footshock over seven days was similar to that found after infusion of CRH by

way of osmotic minipumps over a period of 4-8 days (Bruhn et al., 1984). This suggests that long-term exposure of POMC cells to endogenous CRH during long-term stress might be a factor in induction of POMC gene expression. The present study shows that the elevation of plasma ACTH concentration after application of a stress persists for over 6h and that this is sufficient to stimulate induction of POMC gene expression. The mechanism by which stress induces POMC mRNA changes is unknown but alterations may reflect changes in mRNA formation (e.g. transcription, RNA processing and/or RNA degradation). There are preliminary indications that stress may cause an increase in the in vitro transcription rate of the POMC gene (Eberwine & Roberts, 1984). Plasma concentrations of ACTH at 6h after stress may have been reduced by negative feedback effects of high circulating corticosterone. A number of workers have shown that the inhibition of adrenocortical responses is maximal 2-4h after administration of a single dose of corticosterone (Keller-Wood & Dallman, 1974; Kendall, 1971). POMC gene transcription rates have been shown to change 15 min after glucocorticoid administration, but changes in POMC mRNA content are not detected until much later (Eberwine & Roberts, 1984).

Stimulation of the PVN for 30 min resulted in a smaller elevation of plasma ACTH concentration than ether stress (5- compared with 10-fold, see Fig. 7.7) and the present results suggest that this stimulus is not sufficient to induce changes in POMC mRNA content in the anterior pituitary gland. The PVN contains CRH and AVP cell bodies that project to the median eminence (Tilders et al., 1982; Merchenthaler et al., 1984) where they release their neuropeptides into the hypophysial portal blood

vessels (Horn et al., 1985) and, therefore, stimulation of the PVN should increase the release of CRH and AVP into portal blood. Both CRH and AVP have been demonstrated to stimulate ACTH secretion and it has been reported that long-term administration of ovine CRH can increase the anterior pituitary gland content of POMC mRNA *in vivo* (Bruhn et al., 1984). The increase in POMC mRNA content in the anterior pituitary gland 6h after ether stress followed a 10-fold increase in ACTH secretion. Electrical stimulation of the PVN for 30 min produced a 5-fold increase in ACTH secretion and did not increase POMC mRNA content; unfortunately the high mortality rate of the animals prevented longer stimulation of the PVN.

The Brattleboro rat has been shown to have a 2-fold increase in POMC mRNA content in both genotypes over the isogenic control the Long Evans strain of rats. This was a surprising result given that the ACTH secretogogue, AVP, is thought to be reduced in the heterozygous and absent in the homozygous Brattleboro. However, plasma ACTH and corticosterone concentrations are reduced in Brattleboro rats but this was only significant in the heterozygous Brattleboro rat (see Chapter 5). Lack of AVP results also in diabetes insipidus and Brattleboro rats are continuously searching for water and this could perhaps be regarded as a continuous stress which affects or involves the hypothalamo-pituitary-adrenal system and thereby elevates POMC mRNA content above the values in the Long Evans rats. Injection of dDAVP has been shown to restore urine output in DI rats to control values (e.g. Valtin et al., 1975; Sutherland et al., 1985). Whether dDAVP has 'CRF activity' is not clear. Some groups have reported that dDAVP stimulates ACTH secretion (Pearlmutter et al., 1974; Aizawa et al., 1982) while

others report that dDAVP has no CRF activity (Andersson et al., 1972; Lazlo et al., 1981). After injection with dDAVP in the present study, peripheral plasma concentrations of ACTH and corticosterone were significantly increase in homozygous and heterozygous Brattleboro rats (Chapter 5) and POMC mRNA content was unchanged in both Brattleboro genotypes, suggesting stress caused by water seeking was not responsible for elevated POMC mRNA content in the anterior pituitary gland. These results suggest that dDAVP does indeed have 'CRF activity' but does not affect the synthesis of ACTH. The implications of the elevated POMC mRNA content in Brattleboro rats in relation to control of ACTH and corticosterone release will be further discussed in Chapter 5.

CHAPTER 4

REGULATION OF ACTH SECRETION BY GLUCOCORTICOIDS

4.1 INTRODUCTION

The key role played by the hypothalamus in the regulation of ACTH secretion has been well established (for review, see Chapter 1). The isolation and characterisation of a peptide with potent corticotropin-releasing activity (Vale et al., 1981) and the demonstration of its presence in hypophysial portal blood (Gibbs & Vale, 1982) ended controversy as to whether AVP might have the additional role of the principal CRF of the hypothalamus. Nevertheless, it is clear that both AVP and oxytocin (OT), which are themselves weak ACTH secretagogues, have the ability to potentiate the effect of CRH on the release of ACTH from the anterior pituitary (Gillies et al., 1982; Vale et al., 1983; Gibbs et al., 1984). At concentrations of CRH above which no further increase in ACTH secretion is observed, addition of AVP or OT further elevates ACTH secretion from anterior pituitary cells in culture. This potentiation is observed at concentrations of AVP and OT that are in the range of those found in portal blood (Horn et al., 1985; Zimmerman et al., 1973) and, therefore, it is possible that interactions between AVP or OT and CRH may play a physiological role.

The origin of CRH, AVP and OT and physiology of release have not been clearly defined. Anatomical studies indicate that neurones delivering CRH to hypophysial portal vessels in the median eminence are concentrated in a discrete zone of the parvocellular division of the PVN (Bloom et al., 1982; Merchanthaler et al., 1983; Swanson et al., 1983). In contrast, most OT- and AVP-containing neurones are concentrated in an anatomically distinct magnocellular division that projects to the pars nervosa (Sawchenko & Swanson, 1982; Rhodes et al., 1981), though cells of each type are scattered

through the parvocellular division and project to the external layer of the median eminence (Zimmerman et al., 1977). In colchicine treated animals, a small number of neurones in the magnocellular division of the PVN jointly expressed CRH and OT immunoreactivity, while a very small number of neurones in the parvocellular division stain for both CRH and AVP or OT. This expression of two peptides in individual parvocellular neurones suggests that these peptides may interact in the control of ACTH secretion (Sawchenko et al., 1984b).

Removal of the negative feedback effect of glucocorticoids by adrenalectomy results in marked increases in the transcription of the ACTH gene (Eberwine & Roberts, 1984) and in the synthesis and secretion of ACTH (Birnberg et al., 1983; Dallman et al., 1972) which can be reversed by glucocorticoid treatment. Glucocorticoids have been shown to inhibit ACTH synthesis and secretion by an action at the level of the corticotroph itself (Sayers & Portanova, 1974; Widmaier & Dallman, 1984) and also at the level of the hypothalamus. Several groups have reported that adrenalectomy markedly enhances CRH immunostaining in the PVN (Swanson et al., 1983; Merchenthaler et al., 1983). Vasopressinergic, and to a lesser extent oxytocinergic projections to the median eminence show an enhanced immunoreactivity after adrenalectomy which is reversed by dexamethasone (Stillman et al., 1977). Immunohistochemical double staining techniques have shown adrenalectomy greatly increases the number of neurones in which CRH and AVP are co-localised. Within neurones of the parvocellular region of the PVN more than 70% of CRH cells stained positively for AVP. The distribution of OT-stained cells was unaffected by adrenalectomy

(Sawchenko et al., 1984b). The increase in the number of neurones in which CRH and AVP are co-localised is presumably due to removal of glucocorticoids and, therefore, the presence of AVP in parvocellular neurones in the PVN is presumably affected by the withdrawal of adrenal steroids. The dependence of AVP expression in parvocellular neurones on adrenal steroid withdrawal has been further confirmed by in situ hybridisation studies which showed that the density of specific AVP mRNA increased in the magnocellular portion of the PVN after adrenalectomy and that the distribution of AVP mRNA expanded into the parvocellular region of the PVN. AVP mRNA could be detected in CRH immunoreactive neurones after adrenalectomy but not in control animals. The changes in AVP mRNA content and distribution were prevented by dexamethasone treatment (Davies et al., 1986).

These data suggest that in addition to its neurosecretory role in the neural lobe, AVP may be involved in the regulation of adenohypophysial function after adrenalectomy and, therefore, in the negative feedback control of ACTH secretion. The aim of the present study was to determine the mechanism of negative feedback of glucocorticoids by examining the effect of adrenalectomy on the release of CRH, AVP and OT into hypophysial portal blood and pituitary responsiveness to CRH. In preparation for this study, it was necessary to determine the effect of three different anaesthetics on the elevation of ACTH and corticosterone concentrations during surgery and whether anaesthesia would allow the circadian rhythm of ACTH and corticosterone to be maintained.

4.2 MATERIALS AND METHODS

4.2 1 Animals and Surgery

Adult female **Wistar** COB rats (200-250g body weight) purchased from Charles River U.K. Ltd. (Margate, Kent) were maintained under controlled lighting (light on 0500-1900h) and temperature (22°C) and allowed free access to diet 41B (Oxoid, Basingstoke, Hants) and tap water. Adrenalectomy was carried out under tribromoethanol in two stages as described in 2.2.4. Animals that had undergone adrenalectomy drank 0.9% (w/v) NaCl solution containing 2% glucose and 40mg l^{-1} chlortetracycline hydrochloride. A three week period of recovery was allowed before the animals were used.

Peripheral blood samples were taken from anaesthetised animals with heparinised syringes from the external jugular vein. The samples were immediately mixed with 2000KIU Trasylol/ml blood in chilled plastic tubes before spinning at 1,720 x g for 20 min. Plasma was stored at -40°C for assay. Hypophysial portal blood was collected as described in 2.2.6 during two consecutive 30 min periods. Blood samples were immediately mixed with Trasylol 1000KIU/ml blood in chilled plastic tubes before spinning at 1,720 x g for 20 min and the plasma was stored at -40°C for assay. Hypothalami and anterior pituitary glands were dissected out as described in 2.2.3 and tissue samples were homogenised in 5-10 volumes 2M acetic acid.

4.2.2 Anaesthetics and Drugs

Surgery was carried out under one of the following anaesthetics, all given intraperitoneally:

i) Urethane: Ethyl Carbamate (Sigma Chemicals); 1g kg^{-1} body weight dissolved 1:10 in saline.

ii) Sagatal: Sodium pentobarbitone (May & Baker Ltd.); 36mg kg^{-1} body weight, diluted 1:10 with saline.

iii) Althesin: (Glaxovet Ltd., Uxbridge); 1.0ml $100g^{-1}$ body weight.

Dexamethasone (9α-fluro-16α methyl prednisolone) supplied by Sigma, was stored as a stock solution of 0.9mg ml^{-1} ethanol and diluted 1:10 with saline immediately before use.

Rat corticotrophin-releasing hormone (Peninsula Labs) was dissolved in 0.05N HCl and stored at 0.1mg ml^{-1} and diluted before use in 0.9% (w/v) saline containing 1mg ml^{-1} ascorbic acid and 10mg ml^{-1} bovine serum albumin.

4.2.3 Assays

The concentrations of CRH in plasma and hypothalamic tissue samples were determined as described in 2.3.6. Plasma samples of 25-100μl were extracted with 85% methanol and set up in single aliquots while standards and pools were set up in duplicate. The lower limit of sensitivity ranged from 2-5pg per tube. Tissue samples were homogenised in 200μl Acetic acid and diluted 1:100 in 2M acetic acid. 100μl aliquots were evaporated to dryness, reconstituted in assay buffer and assayed as described in 2.3.6.

OT and AVP concentrations were determined as described in 2.6. Plasma samples of 10μl were set up in single aliquots and standards in duplicate. The lower limit of sensitivity ranged from 4-6pg per tube for OT and 2-3pg per tube for AVP.

Concentrations of ACTH in plasma and anterior pituitary tissue samples were determined as described in 2.4. Plasma samples of 10-50µl were set up in duplicate and standards and pools in triplicate. The lower limit of sensitivity ranged from 5-10pg ml^{-1}. Pituitary samples were homogenised in 100µl 2M acetic acid and then diluted 1:20,000 in 2M acetic acid. Aliquots from the diluted extract of 10 and 25µl were taken in duplicate, evaporated to dryness and assayed as above.

Corticosterone concentrations were measured as described in 2.5. Single samples of peripheral plasma (20-50µl volume) were extracted with ether and aliquots of the extract dried down and set up in duplicate in the assay. Standards were diluted in methanol dried down and set up in duplicate in the assay. Crossreactivity of the antiserum to dexamethasone was less than 0.1%.

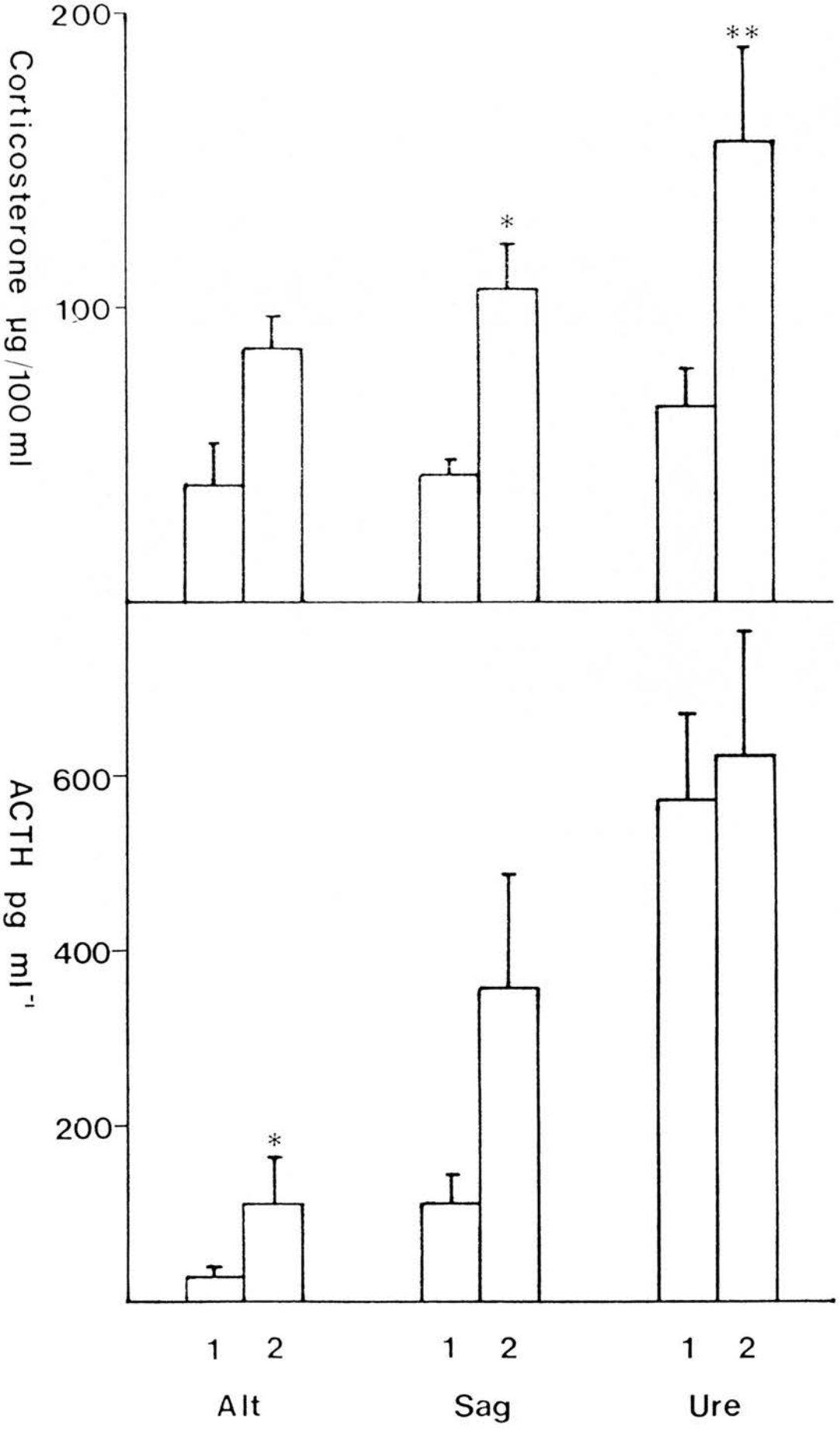
4.3 RESULTS

4.3.1 Anaesthetics

Six groups of six female Wistar rats were anaethetised with either urethane, saqatal or althesin and peripheral blood collected from the external jugular vein. Samples were taken at 20 min intervals between 0900-1000h and between 1700-1800h and assayed for ACTH and corticosterone. Figure 4.1 shows ACTH and corticosterone concentrations at both time points under the three anaesthetics, taking the late afternoon peak and the lowest morning values for each anaesthetic. The circadian rhythm of ACTH and corticosterone was maintained under anaesthesia with althesin; plasma concentrations of ACTH increased 4-fold and corticosterone 2-fold in

Figure 4.1 Effect of anaesthesia on the circadian rhythm of ACTH and corticosterone in plasma.

Mean (± S.E.M.; n = 6) concentrations of ACTH (pg ml^{-1}) and corticosterone (μg.100ml) in peripheral plasma from female Wistar rats at 09.20h and 17.40h (1 and 2 respectively) under anaesthesia with althesin (Alt), sagatal (Sag) and urethane (Ure). Blood samples were withdrawn from the external jugular vein. *$p < 0.01$ and ** $p < 0.05$ when compared with plasma concentrations at 09.20h (Student's t-test).


Corticosterone µg/100 ml
200
100
**
*
ACTH pg ml⁻¹
600
400
200
*
1 2
1 2
1 2
Alt
Sag
Ure

the afternoon compared with the morning although the difference in corticosterone concentration was not significant. Similar results were obtained with sagatal; plasma ACTH concentrations increased 3-fold and corticosterone 2-fold with only the difference in corticosterone concentrations being significant. The plasma concentrations of ACTH were considerably higher under urethane anaesthesia compared with the values in animals under the other anaesthetics and the circadian rhythm of ACTH was abolished under urethane anaesthesia. Although ACTH concentrations under sagatal anaesthesia were elevated above those with althesin anaesthesia, sagatal was chosen as the anaesthetic for the remainder of experiments because the survival rate was much higher with sagatal compared with althesin anaesthesia.

4.3.2 Hypophysial blood collection

Results are expressed as the concentration of CRH, AVP and OT in hypophysial portal plasma and also as content (concentration x volume in 30 min) of CRH, AVP and OT in hypophysial portal plasma. Expression as content allows for correction of different volumes of plasma collected and is the total amount of hormone secreted into portal blood during the 30 min collection period (Fink & Jameson, 1976).

4.3.3 Effect of Adrenalectomy on ACTH and corticosterone concentrations in peripheral plasma and the anterior pituitary gland

Peripheral concentrations of ACTH and corticosterone were measured in control female Wister COB rats and in female Wister COB

rats three weeks after total adrenalectomy. Animals were anaesthetised with sagatal, the pituitary stalk exposed and peripheral samples taken from the external jugular vein immediately before cutting the pituitary stalk. Fig. 4.2 shows that adrenalectomy significantly increased the concentration of ACTH 4-fold. Whether adrenalectomy was complete was confirmed by the low concentrations of corticosterone in plasma and by the absence of adrenal tissue at autopsy. The anterior pituitary content of ACTH was significantly reduced ($p < 0.001$; $n = 5$) from 11.5 ± 1.1μg per pituitary to 5.83 ± 0.8μg per pituitary by adrenalectomy. In adrenalectomised animals injected with dexamethasone, 0.5mg kg^{-1} body weight 2.5h before blood collection, the ACTH concentration was decreased 2-fold (Fig. 4.2). The pituitary content of ACTH in adrenalectomised rats treated with dexamethasone was 13.6 ± 0.9μg/pituitary gland which is significantly greater ($p < 0.001$; $n = 5$) than the value of 5.83 ± 0.8μg/pituitary gland in untreated adrenalectomised rats.

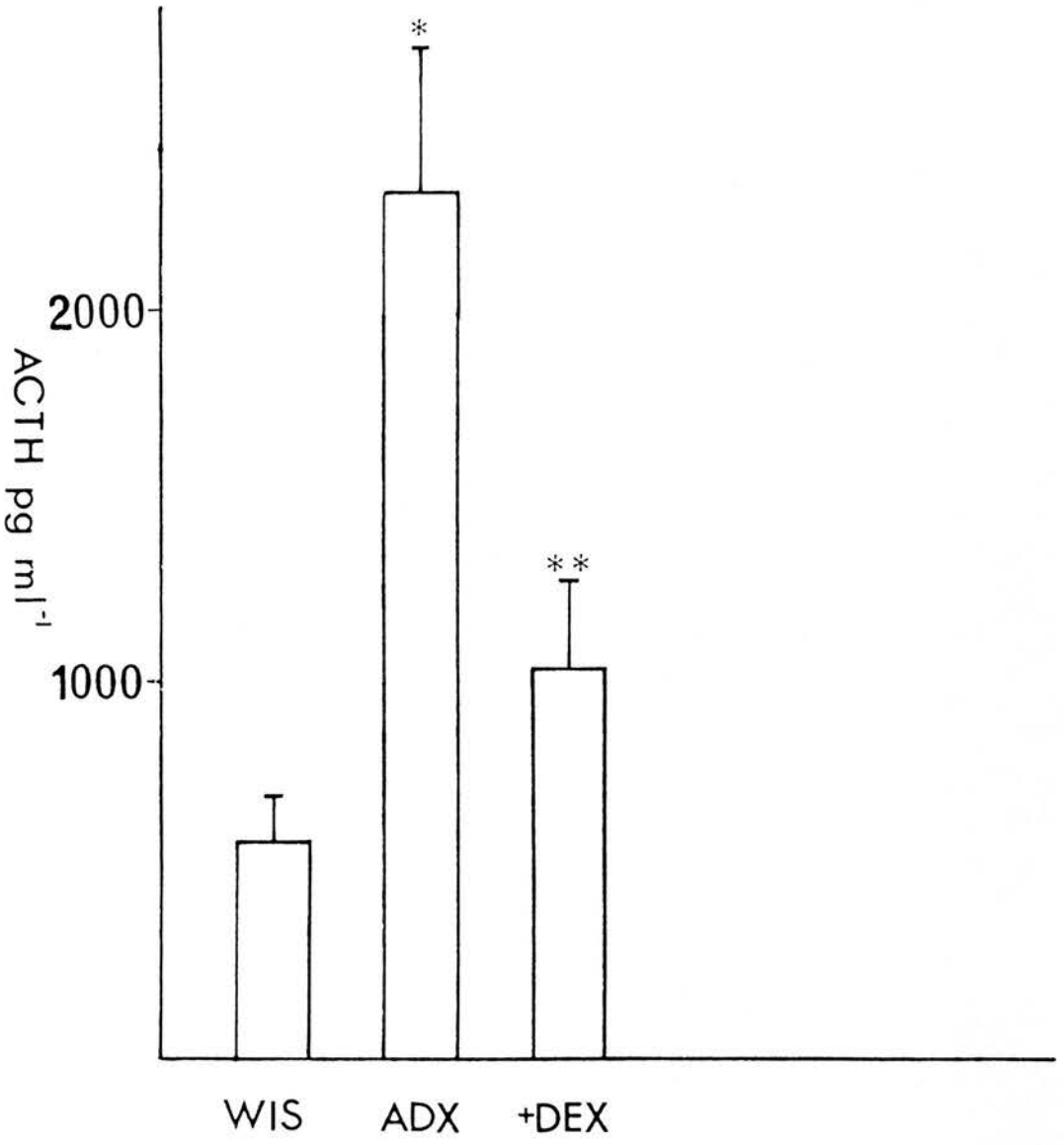
4.3.4 Effect of adrenalectomy on concentrations of CRH, AVP and OT in hypophysial portal plasma

Fig 4.3 shows that adrenalectomy three weeks before collection of hypophysial portal blood produced a significant 5-fold increase in the CRH content in portal plasma when compared with the content of CRH in portal plasma of intact control animals. Dexamethasone injection had no significant effect on the concentration or content of CRH in portal plasma in adrenalectomised animals when compared with the values in untreated adrenalectomised animals (Fig. 4.3).

The concentrations and contents of AVP in hypophysial portal plasma were significantly increased in adrenalectomised rats when

Figure 4.2 Effect of adrenalectomy on plasma ACTH and corticosterone concentrations

Mean (± S.E.M.; n = 10) concentration of a) ACTH and b) corticosterone in peripheral plasma from intact female Wistar rats (WIS), Wistar rats that had been adrenalectomised for three weeks (ADX) and adrenalectomised rats treated with dexamethasone 2.5h before blood collection. Blood samples were taken from the external jugular vein after exposure but before section of the pituitary stalk. *$p < 0.005$ when compared with intact Wistar rats and **$p < 0.001$ when compared with untreated adrenalectomised animals. ***$p < 0.0001$ when compared with intact Wistar rats (Student's t-test).


ACTH pg ml⁻¹
2000
1000
*
**
WIS
ADX
+DEX

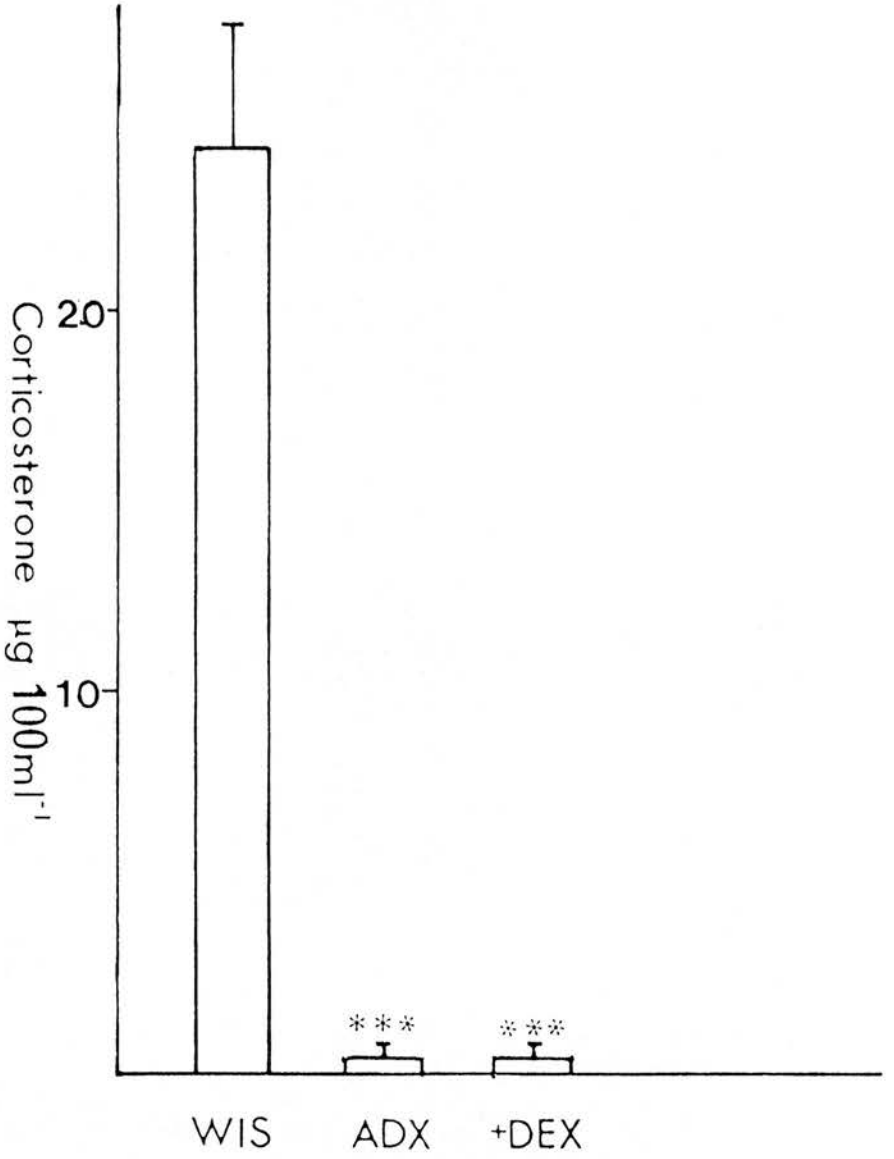
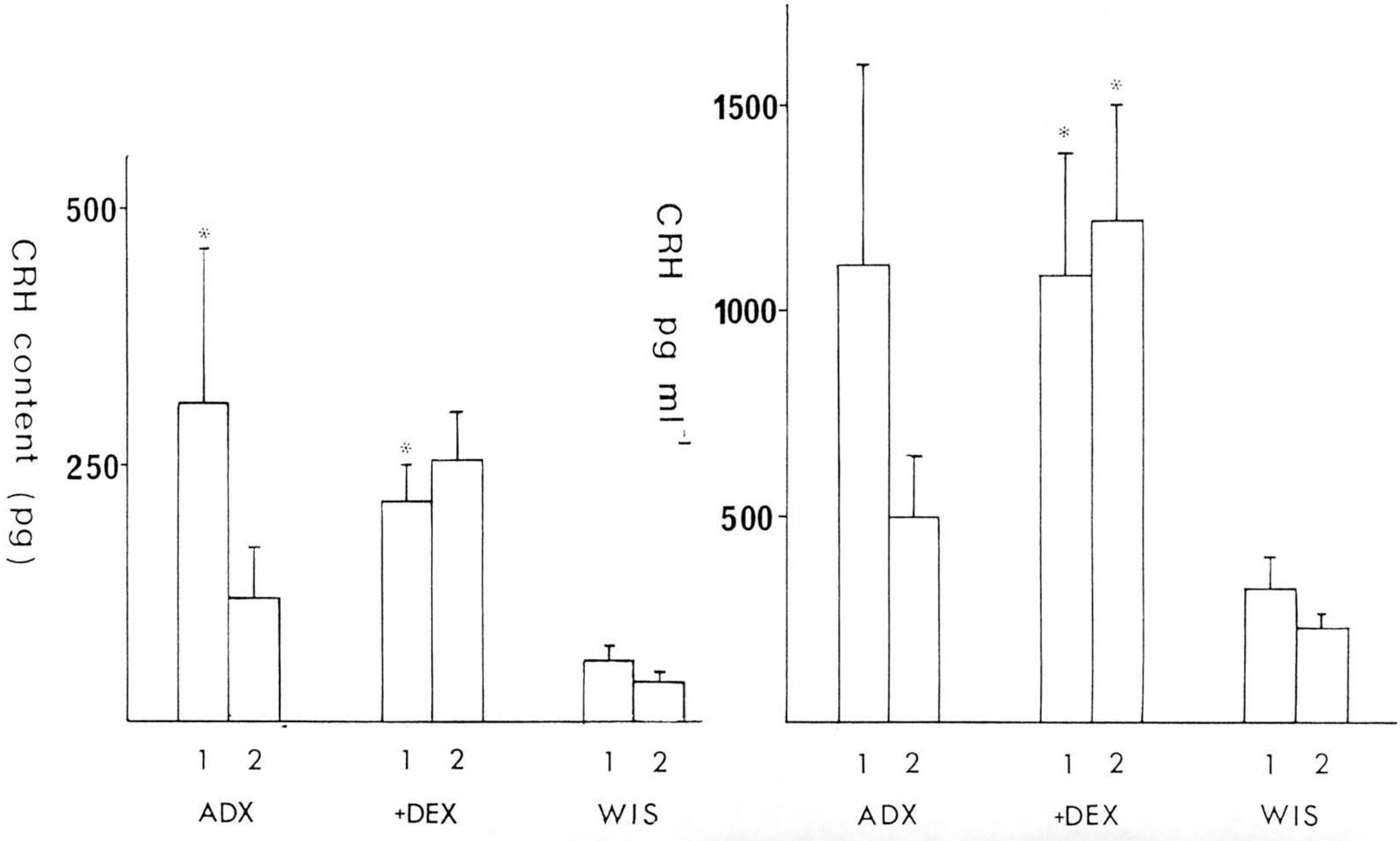

Corticosterone µg 100ml⁻¹
20
10
***
***
WIS
ADX
+DEX

Figure 4.3 The effect of adrenalectomy on CRH release into hypophysial portal blood

Mean (± S.E.M.; n = 10) concentration (pg ml^{-1}) and content (pg/30 min) of corticotrophin-releasing hormone (CRH) in hypophysial portal blood in intact female Wistar rats (WIS), Wistar rats that had been adrenalectomised for three weeks (ADX) and adrenalectomised rats injected with dexamethasone 2.5h before blood collection (+ DEX). Hypophysial portal blood was collected over two consecutive 30 min periods (1 and 2). *$p < 0.05$ when compared with intact Wistar rats (Student's t-test).


CRH content (pg)
500
250
CRH pg ml⁻¹
1500
1000
500
*
1 2
ADX
+DEX
WIS

compared with the values in control animals. AVP content and concentration were, respectively, 4- and 5-fold greater in adrenalectomised than in control rats (Fig. 4.4). Dexamethasone injection resulted in a 2-fold decrease in concentration and content of AVP when compared with the values in untreated adrenalectomised rats although the concentrations did not return to the values in intact control rats (Fig. 4.4).

There was no significant difference between the concentration and content of OT in hypophysial portal blood in adrenalectomised rats when compared with control rats (Fig. 4.5). Dexamethasone injection also had no significant effect on OT release into hypophysial portal blood.

4.3.5 Pituitary responsiveness to CRH after adrenalectomy

Female Wistar COB rats, adrenalectomised three weeks previously, were injected with either saline or 0.5mg kg^{-1} body weight dexamethasone and anaesthetised with sagatal. Three hours after injection, the external jugular vein was exposed and an initial peripheral blood sample collected. This was followed by intravenous injection of either saline, 0.1µg CRH or 3µg CRH and blood samples collected 5, 15 and 60 min after injection. Fig 4.6 shows injection of 0.1µg CRH to saline treated animals produced a 2-fold increase in the plasma ACTH concentration above the initial ACTH concentration when compared with injection of saline which increased ACTH concentrations above control values by 20%. Injection of 3µg CRH produced a 4-fold increase in the plasma ACTH concentrations. Dexamethasone injection decreased initial plasma ACTH concentrations 10-fold and significantly reduced the ACTH

Figure 4.4 The effect of adrenalectomy on release of vasopressin (AVP) into hypophysial portal blood

Mean (± S.E.M.; n = 10) concentration (ng ml^{-1}) and content (ng/30 min) of vasopressin (AVP) in hypophysial portal blood in intact female Wistar rats (WIS), Wistar rats that had been adrenalectomised for three weeks (ADX) and adrenalectomised rats injected with dexamethasone 2.5h before blood collection (+ DEX). Hypophysial portal blood was collected over two consecutive 30 min periods (1 and 2). *$p < 0.001$ when compared with intact Wistar rats **$p < 0.02$ when compared with untreated adrenalectomised rats (Student's t-test).

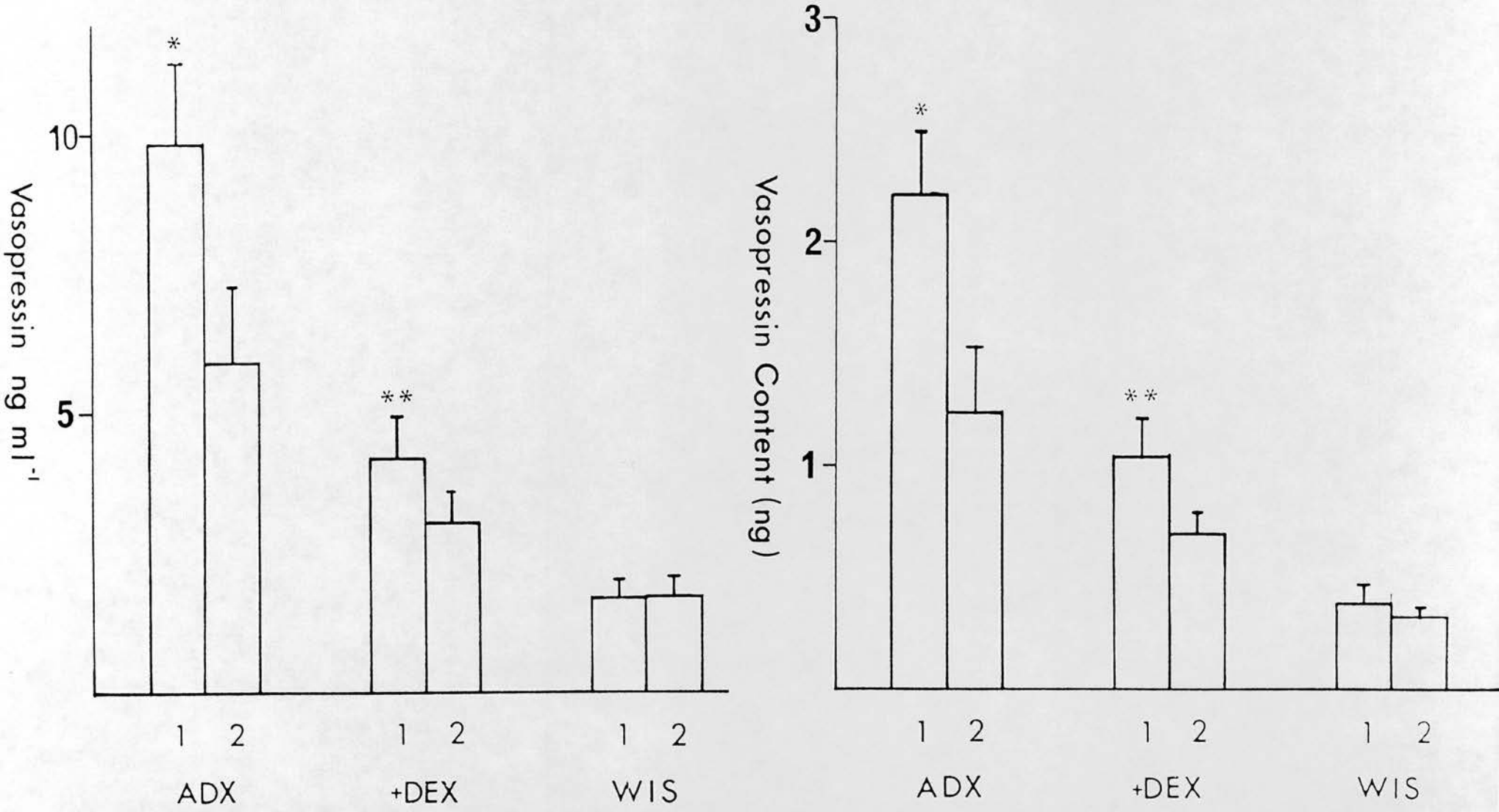
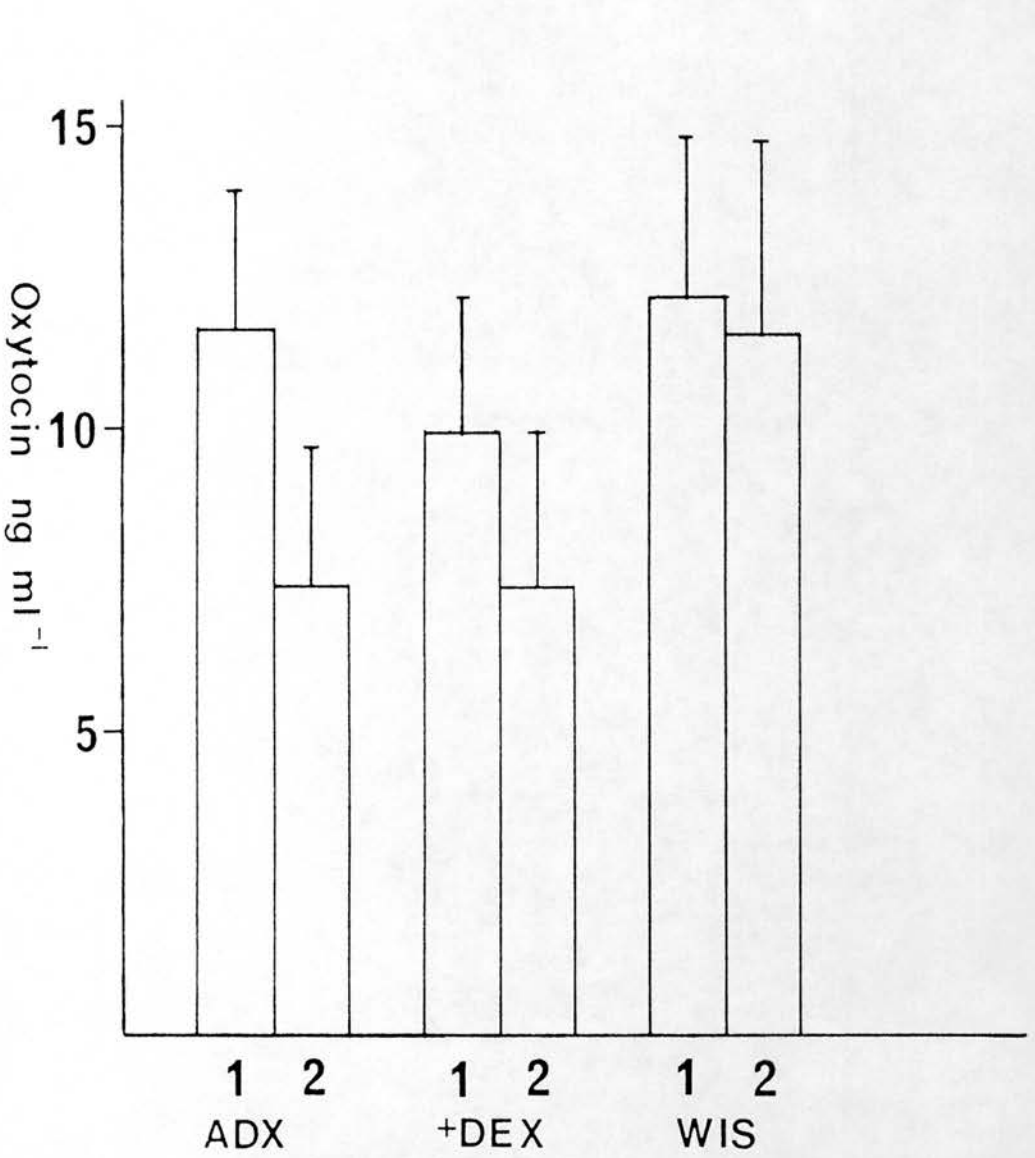
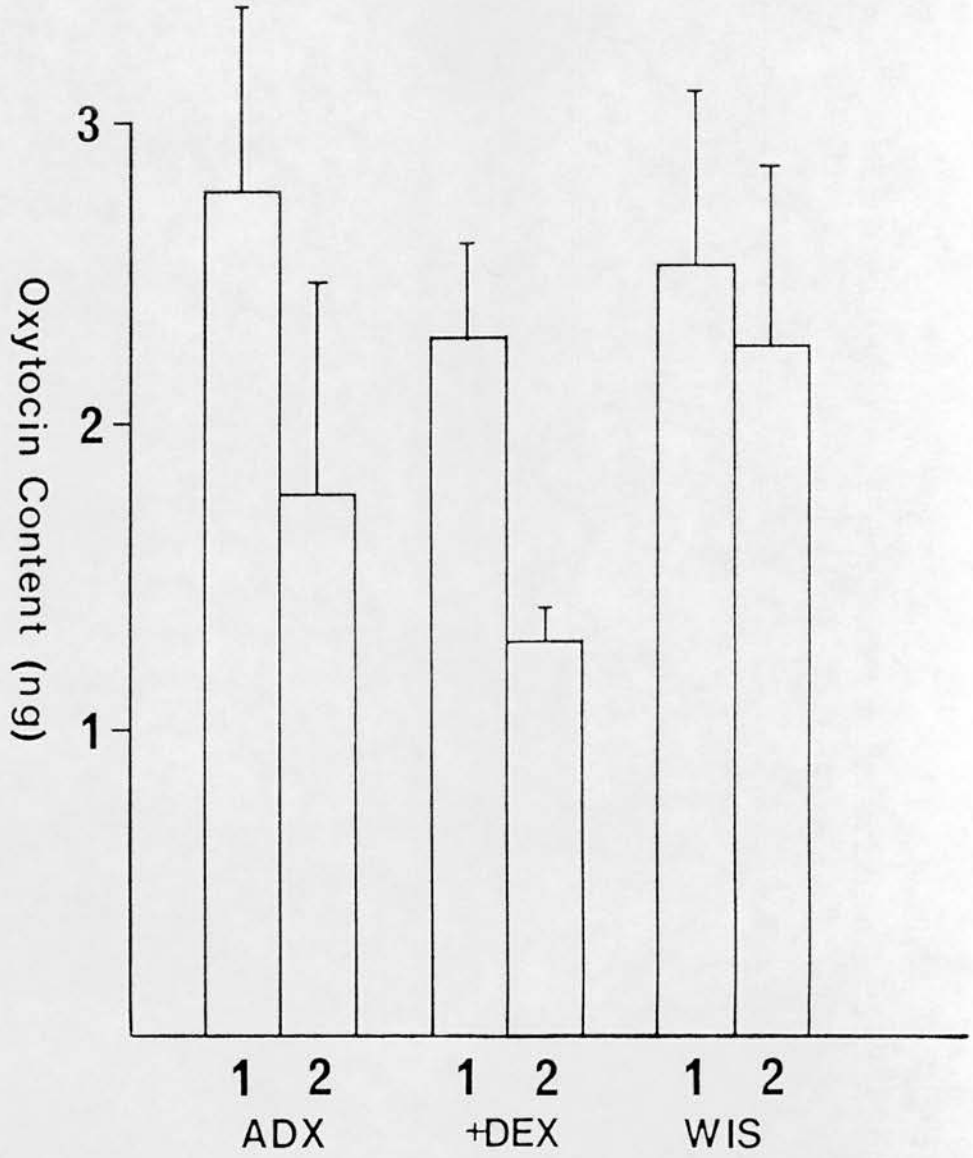

Vasopressin ng ml⁻¹
10
5
*
**
1 2
1 2
1 2
ADX
+DEX
WIS
Vasopressin Content (ng)
3
2
1
*
**
1 2
1 2
1 2
ADX
+DEX
WIS

Figure 4.5 The effect of adrenalectomy on release of oxytocin into hypophysial portal blood

Mean (± S.E.M.; n = 10) concentration (ng ml^{-1}) and content (ng/30 min) of oxytocin in hypophysial portal blood in intact female Wistar rats (WIS), Wistar rats that had been adrenalectomised for three weeks (ADX) and adrenalectomised rats injected with dexamethasone 2.5h before blood collection (+ DEX). Hypophysial portal blood was collected over two consecutive 30 min periods (1 and 2).


Oxytocin ng ml⁻¹
15
10
5
1 2
ADX
1 2
+DEX
1 2
WIS


Oxytocin Content (ng)
3
2
1
1 2
ADX
1 2
+DEX
1 2
WIS

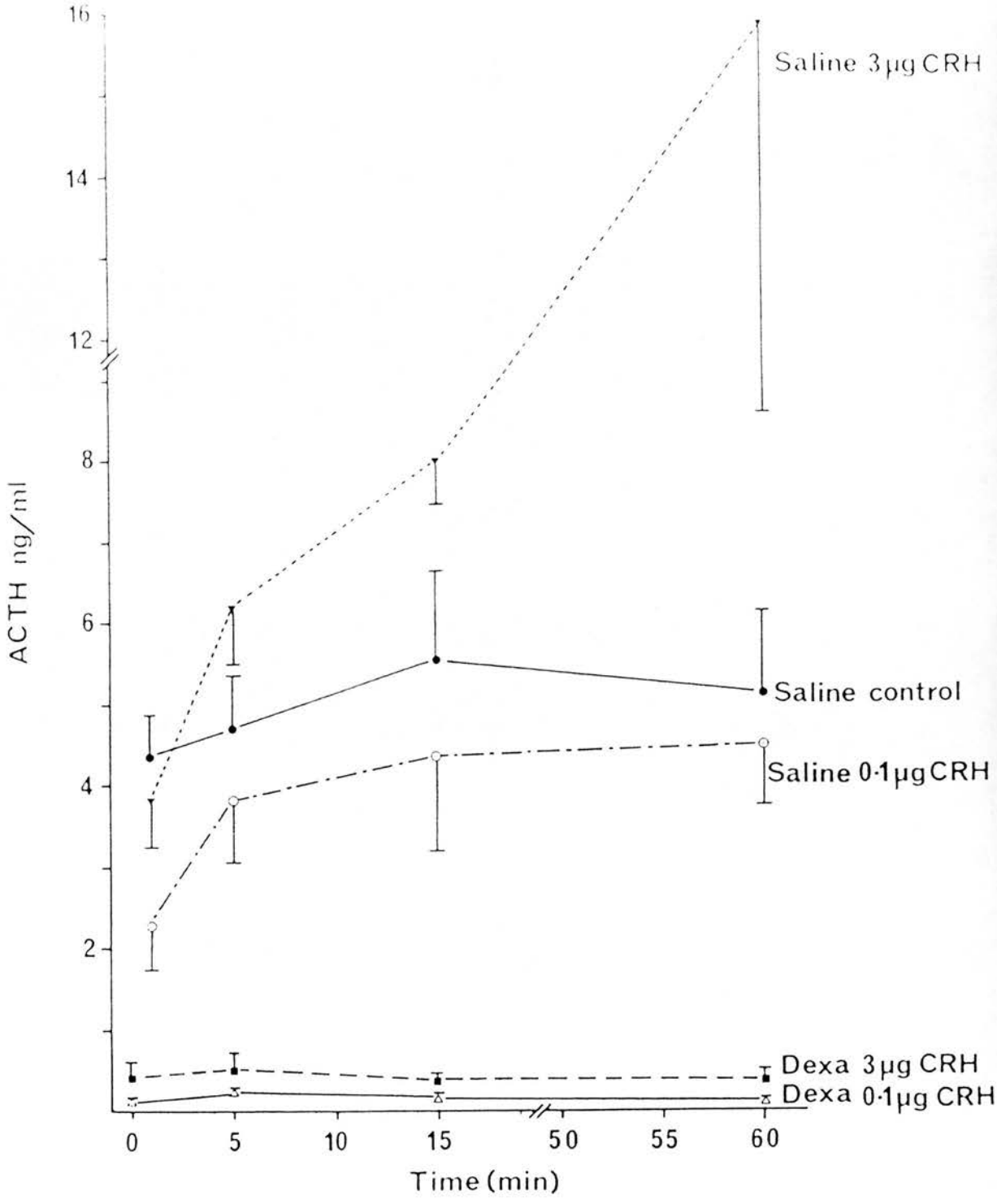


Figure 4.6 Pituitary responsiveness to CRH after adrenalectomy

Mean (± S.E.M.; n = 5) concentration of ACTH (pg/ml) in peripheral plasma from adrenalectomised female Wistar rats. Rats were injected with either dexamethasone (Dexa) or saline 3h before blood samples were withdrawn from the external jugular vein (time 0). Immediately after this, the rats were challenged with either saline, 0.1µg CRH or 3µg CRH and blood samples collected 5, 15 and 60 min after the challenge.

response to injections of either 0.1 or 3.0µg CRH. Due to the substantial differences in baseline ACTH concentrations between the dexamethasone and the saline treated rats, the results are expressed in Fig. 4.7 as a percentage increase above the initial plasma ACTH concentration. Figure 4.7 shows that there was a dose response effect to CRH in saline-treated adrenalectomised rats and that while the dexamethasone treated adrenalectomised rats did respond to 3µg CRH, the response was significantly lower than in the saline-treated adrenalectomised rats.

4.4 DISCUSSION

The choice of anaesthetic has been shown previously to have important consequences. For example, althesin anaesthesia does not block the spontaneous ovulatory surge of LH or the associated surge of LHRH, in contrast, sagatal and urethane anaesthesia block both these surges (Sarkar et al., 1976; Fink & Jamieson, 1976; Sherwood et al., 1980). Urethane has been shown to elevate SS in portal blood compared with sagatal or althesin anaesthesia (Chihara et al., 1976). Also, the release of TRH into portal blood during the afternoon of pro-oestrous was higher with althesin than with sagatal or urethane (Fink et al., 1982). ACTH and corticosterone are secreted in a circadian fashion (Chiappa & Fink, 1977). It was necessary to use an anaesthetic that would not substantially elevate the secretion of these hormones or abolish the circadian rhythm, otherwise changes in the release of these hormones and of their hypothalamic releasing factors could be masked. In the present study, urethane anaesthesia elevated plasma ACTH and corticosterone concentrations above control, sagatal and althesin

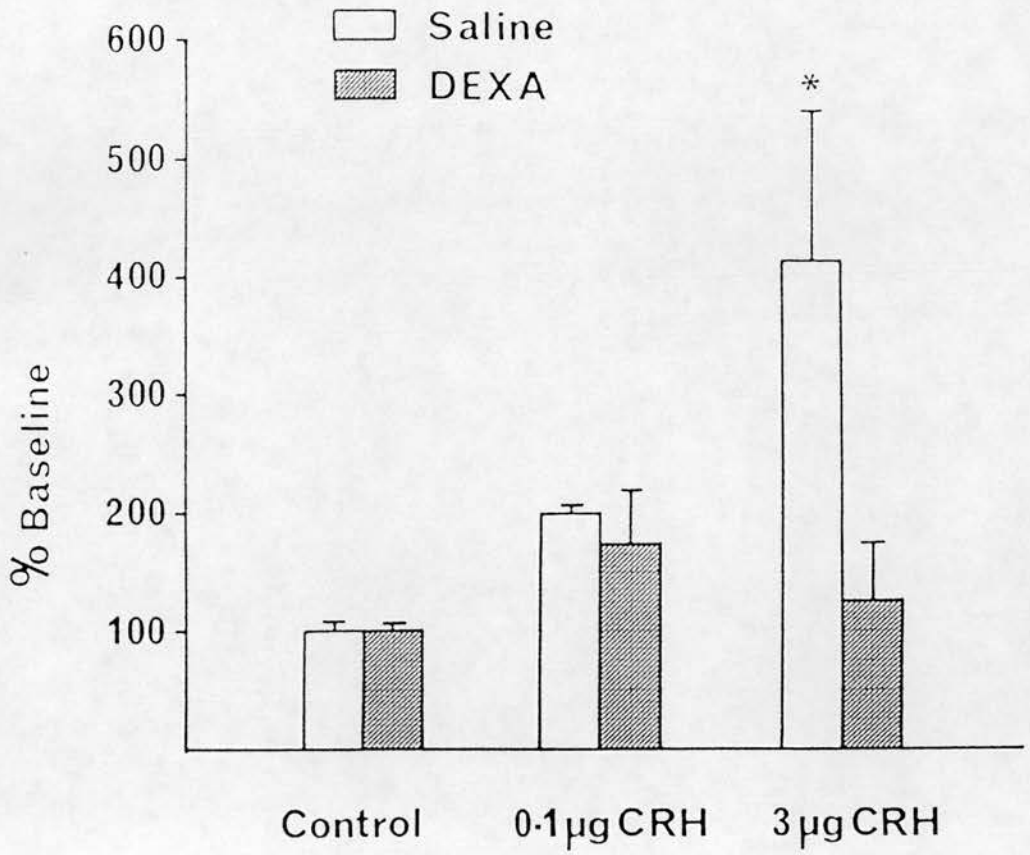


Figure 4.7 Pituitary responsiveness to CRH after adrenalectomy

Mean (± S.E.M.; n = 5) of the percentage increase over time 0 in plasma concentrations of ACTH 60 min after the challenge of saline, 0.1 µg CRH or 3 µg CRH. Female Wistar rats that had been adrenalectomised for three weeks were pretreated 3 h before blood collection with either saline or dexamethasone (DEXA). *P < 0.05 when compared to the dexamethasone treated rats (Student's t-test).

levels and abolished the circadian rhythm ACTH. These results agree with the work of Hamstra et al. (1984). Hamstra proposed that urethane was stimulating the cholinergic system as the increased secretion of corticosterone could be reversed by atropine. The present study demonstrates that the circadian rhythm of corticosterone is maintained with sagatal anaesthesia but not with althesin, this is in agreement with Honma et al. (1984) and Dunn & Doray (1984). However, the opposite applies to the ACTH rhythm with althesin and not sagatal maintaining the circadian rhythm. From these results, there was very little difference between **althesin** and sagatal anaesthesia as neither anaesthetic substantially elevated the secretion of ACTH and corticosterone. Sagatal was used routinely as it gave a higher survival rate than althesin.

These results, using sagatal anaesthesia, show that interruption of the negative feedback effect of glucocorticoids by adrenalectomy results in a significant increase in both CRH and AVP release into hypophysial portal blood. The increased release of AVP, but not CRH, can be significantly reduced by dexamethasone injection. Adrenalectomy resulted in a marked increase in ACTH synthesis as shown by increased content of POMC mRNA in the anterior pituitary (see Chapter 3). Secretion was reduced to that in intact rats by dexamethasone administration 2.5h before blood collection and this finding agrees with that of other reports (Eberwine & Roberts, 1984; Birnberg et al., 1983). However, after three weeks of adrenalectomy, the anterior pituitary content of ACTH was significantly reduced when compared with the values in control animals. The finding of reduced pituitary ACTH content differs

from the findings of Buckingham & Hodges (1974) and Dallman et al. (1972), who found an increase in pituitary content three weeks after adrenalectomy. The pituitary ACTH contents in the present study suggest that the increase in the synthesis of ACTH in the anterior pituitary gland after adrenalectomy (as discussed in chapter 3) does not compensate for the increased secretion of ACTH into peripheral plasma and, therefore, results in a depletion of the content of ACTH in the anterior pituitary gland.

The mechanism by which the corticotroph responds to adrenalectomy has been the focus of many recent studies. Dallman et al. (1985) have shown that in animals in which anterolateral hypothalamic deafferentation has been carried out, the corticotroph was unable to respond to adrenalectomy by increasing ACTH synthesis and secretion. The findings of Dallman et al. (1985) suggest that hypothalamic drive plays an important role in glucocorticoid control of ACTH secretion. Wynn et al. (1985) have demonstrated a down-regulation of receptors for CRH in the anterior pituitary gland after adrenalectomy which was accompanied by a comparable decrease in CRH stimulated adenylate cyclase activity in the anterior pituitary gland. The ability of the corticotroph to maintain increased ACTH release in spite of down-regulation of the CRH receptors indicates that elevated ACTH secretion can be maintained by occupancy and activation of a small number of CRH receptors and also suggests that synergistic interactions between CRH and other regulators of ACTH release may contribute to the substantial increase in ACTH secretion that follows adrenalectomy. The interactions between CRH and other peptides in the regulation of ACTH secretion has been confirmed by the present study, in that

increased release of CRH and AVP into hypophysial portal blood was found to correlate with increased plasma concentrations of ACTH. OT release remained unaffected by adrenalectomy and this is in agreement with the immunohistochemical studies which showed that no increase in the distribution or density of OT staining occurred after adrenalectomy (Sawchenko et al., 1984a). The ratio of CRH:AVP in hypophysial portal blood after adrenalectomy was the same as in control animals; that is, the portal blood content of both peptides increased 5-fold, suggesting that both peptides are of similar importance in controlling the ACTH response to adrenalectomy. The present result differs from that of Holmes et al. (1986) who found that vasopressin release from the median eminence *in vitro* increased 3- to 4-fold more than CRH release after adrenalectomy. Holmes et al., (1986) suggested that AVP was the predominant ACTH releasing stimulus in adrenalectomised rats. However, the results of Holmes et al. (1986) were based on experiments on tissue containing nerve terminals isolated from their cell bodies and are therefore unlikely to represent the situation *in vivo* and are difficult to compare with the present *in vivo* studies.

Previous reports have found CRH-like activity in the hypothalamus, as measured by bioassay (Krieger et al., 1977) or radioimmunoassay (Suda et al., 1983), increases after adrenalectomy and the increase in CRH-like activity is consistent with the present data. On the other hand, the effects of replacement with glucocorticoids at the pituitary and hypothalamic level have been less consistent. There are known to be at least three distinct time domains in which negative feedback by glucocorticoids on ACTH secretion are exerted: fast (within seconds to minutes),

intermediate (over 2-10h) and slow (over hours to days, for details see section 1.3.3). Thus, interpretation of results of an *in vivo* experiment are dependent on the timing of the experiment relative to the glucocorticoid signal. In the present study, the timing of corticosteroid treatment represents an intermediate level of feedback. Administration of dexamethasone 2.5-3h before blood collection was chosen because a number of studies have indicated that maximal inhibition of adrenocortical function occurs between 2h and 4h after administration of a single dose of dexamethasone (Takebe et al., 1971; Jones et al., 1974a). Replacement of glucocorticoids in adrenalectomised animals with dexamethasone in the present study did not reverse the increase observed in CRH release into portal blood but did decrease the release of AVP. This is contrary to what has been reported by other groups who have found that an intermediate level of feedback decreases the release and synthesis of CRF (Vermes et al., 1977; Hillhouse & Jones, 1976; Jones & Hillhouse, 1976). However, these discrepancies can be explained, in part, by their use of bioassay to determine changes in CRF release. Bioassay would be unable to differentiate between CRH and AVP, thereby masking the differential regulation of the two peptides.

The effect of long-term glucocorticoid feedback on specific CRH mRNA and specific AVP mRNA in the hypothalamus has been examined by Northern blot analysis of hypothalamic mRNA seven days after the removal of glucocorticoids by adrenalectomy. Hypothalamic content of both CRH mRNA (Jungami et al., 1985) and AVP mRNA (Davies et al., 1986) were found to be increased seven days after adrenalectomy suggesting that the increased release of CRH and AVP into portal

blood observed in the present study is the result of increased synthesis of the precursor proteins. Replacement of glucocorticoids in adrenalectomised rats by injection of dexamethasone for seven days reversed the increase observed after adrenalectomy in hypothalamic content of both CRH mRNA (Jungami et al., 1985) and AVP mRNA (Davies et al., 1986) However, in the present study, restoration of 'intermediate' feedback in adrenalectomised rats reduced the release of AVP into portal blood but had no effect on the release of CRH into portal blood when compared to untreated adrenalectomised rats, suggesting that at the intermediate level of feedback, there is differential regulation of AVP and CRH release by glucocorticoids. Differential regulation of AVP and CRH has also been observed in response to insulin-induced hypoglycemia where the AVP, but not the CRH concentration, increases in portal blood (Plotsky et al., 1985c) and in the response to haemorrhage, where CRH acts as the predominant regulatory agent (Plotsky et al., 1985b).

As mentioned above, after adrenalectomy, CRH and AVP are co-localised in the parvocellular division of the PVN and, therefore, it seems reasonable to suggest that the AVP and CRH released into portal blood after adrenalectomy originate from the same nerve terminals. However, the differential regulation of CRH and AVP release by glucocorticoids would argue against this. In the parvocellular region of the PVN only 70% of CRH cells stain for AVP after adrenalectomy and, therefore, it appears that the two CRH cell types in the parvocellular region are differentially regulated by glucocorticoids.

In adrenalectomised animals the secretion of CRH is not reduced

by restoration of glucocorticoid feedback for 3h, however, the effectiveness of CRH in stimulating ACTH secretion from the anterior pituitary gland is dramatically reduced. The results obtained in the present study show that the administration of glucocorticoids to adrenalectomised rats 3h previously, drastically reduced the responsiveness of the pituitary gland to exogenous synthetic CRH and this is in agreement with the results obtained by Rivier et al. (1982) in the intact rat.

In conclusion, removal of the negative feedback of glucocorticoids by adrenalectomy results in an increase in ACTH secretion from the anterior pituitary gland which is a result of an increase in the release of CRH and AVP into hypophysial portal blood. Restoration of glucocorticoid feedback for an 'intermediate' time period reduced ACTH secretion into peripheral plasma by a combination of two factors (1) a reduction in the release of AVP into portal blood and (2) reduced pituitary responsiveness to CRH. The results of the present study indicate that glucocorticoid feedback acts both at the hypothalamic and at the pituitary level to influence ACTH secretion.

CHAPTER 5

REGULATION OF ACTH SECRETION IN THE BRATTLEBORO RAT

5.1 INTRODUCTION

The defect in the hormone secretion of the Brattleboro rat was first discovered when it was noted that Brattleboro rats had a urine output each day that was equivalent to greater than 70% of its body weight, compared to 3% in the Long Evans strain, the strain from which Brattleboro rats are derived. The abnormality in heterozygous Brattleboro rats is intermediate between that of homozygous Brattleboro rats and the normal, Long-Evans rats. The first and major endocrine abnormality to be described in the Brattleboro rat was its deficiency in the neurohypophysial peptides. The lack of AVP results in diabetes insipidus and Brattleboro rats are constantly searching for water. Not only was AVP found to be absent in the posterior pituitary gland of homozygous Brattleboro rat but the posterior pituitary content of OT was also less in Brattleboro rats than in control Long-Evans rats (Sokol& Valtin, 1965; Valtin et al., 1965). However, peripheral plasma concentrations of OT were shown to be higher in homozygous Brattleboro rats than in control rats suggesting reduced pituitary content was a result of increased secretion (Edwards et al., 1982). A partial AVP deficiency was reported in heterozygous Brattleboro rats in posterior pituitary lobe content and peripheral plasma concentrations (Sokol & Valtin, 1965; Moses & Miller, 1970). Analysis of the pedigree of these Brattleboro rats led to the conclusion that the defect in the Brattleboro rat strain is inherited as an autosomal semirecessive trait at a single gene locus (Valtin et al., 1975). Horn et al. (1985) found that AVP was undetectable in hypophysial portal plasma from the homozygous Brattleboro while heterozygous Brattleboro rats had reduced

concentrations of AVP in portal blood when compared to the Wistar. Concentrations of OT in portal blood were significantly higher in both strains of Brattleboro than in the Wistar rat (Horn et al., 1985). The AVP deficit in Brattleboro rats appears to be due to a single base deletion in exon B of the protein coding region of the AVP precursor (Schmale & Richter, 1984). The deletion leads to an 'out-of-frame' reading sequence and loss of both the glycosylation site and the normal temination site with the possible consequence of impeded AVP-mRNA translation (Schmale & Richter, 1984). However, in the homozygous Brattleboro rat, immunoreactive AVP has been demonstrated in the ovaries (Lim et al., 1984) and in the adrenal gland (Nussey et al., 1984) suggesting that the homozygous Brattleboro is not entirely deficient in AVP. Very low concentrations of immunoreactive AVP have also been found in the anterior pituitary gland and hypothalamus of the homozygous Brattleboro rat (Nussey et al., 1984; Lolait et al., 1986), and Mezey et al. (1986) have shown AVP and C-terminal propressophysin immunoreactivity in the PVN, supraoptic nuclei and in the posterior pituitary of homozygous Brattleboro rats suggesting that homozygous Brattleboro rats make small amounts of the normal AVP precursor.

AVP and possibly OT are weak ACTH secretagogues and also have the ability to potentiate the effect of CRH on the release of ACTH from the anterior pituitary gland (see Chapter 1). Immunocytochemical staining has shown that CRH is located in the PVN, supraoptic nuclei, median eminence and in the posterior pituitary gland of both Long-Evans and homozygous Brattleboro rats. Hypothalamic staining for CRH in the Brattleboro rat was weaker than in the Long Evans rat (Burlet et al., 1983).

Therefore, many groups have examined the effect of the Brattleboro rats hormonal deficiencies on ACTH and corticosterone concentrations in peripheral plasma. Plasma ACTH and corticosterone concentrations in homozygous Brattleboro rats have been reported to be similar, higher and lower than those in Long Evans rats (Arimura et al., 1967; Allen et al., 1982; Ixart et al., 1982; Buckingham, 1982; Conte-Devolx et al., 1982; Laulin et al., 1985). These conflicting results may be due, in part, to differences in resting conditions and treatment of the animals.

The aim of the present study was to measure the release of ACTH secretagogues, CRH, AVP and OT into hypophysial portal blood in Long Evans, Wistar and Brattleboro rats. The release of CRH, AVP and OT was correlated with peripheral plasma concentrations of ACTH and corticosterone. In Brattleboro rats, the adrenocortical response to injection of the AVP analogue, dDAVP was also examined.

5.2. MATERIALS AND METHODS

5.2.1 Animals and Surgery

Adult female Wistar COB and Long Evans rats purchased from Charles River U.K. ltd. (Margate, Kent) and Brattleboro rats, bred in the Department of Pharmacology (200-250g body weight), were all maintained under controlled lighting (lights on 0500-1900h) and temperature (22°C) and allowed free access to diet 41B (Oxoid, Basingstoke, Hants) and tap water. Brattleboro rats were housed singly in metabolic cages and urine output was measured over a 24h period. Animals that passed an amount of urine equal or greater than 75% of their body weight were classified as homozygous and

those that passed less than 75%, as heterozygous Brattleboro rats. Confirmation of hormonal phenotype was confirmed at the end of the experiment when the pituitary gland was removed and assayed for AVP.

Rats were removed individually from their cages and decapitated within 1 min. Trunk blood was collected into chilled heparinised plastic tubes containing Trasylol 200KIU/ml blood. Tubes were mixed before spinning at 1,720 x g for 20 min. Plasma was stored at -40°C before assay. Hypophysial portal blood was collected, under anaesthesia with sagatal as described in 2.2.6, over two consecutive 30 min periods. Blood samples were immediately mixed with Trasylol (1000KIU/ml blood) in chilled plastic tubes before spinning at 1,720 x g for 20 min at 4°C, plasma was stored at -40°C. Hypothalamic and anterior pituitary glands were dissected out as described in 2.2.3 and tissue samples homogenised in 5-10 volumes of 2M acetic acid.

dDAVP (10µg/ml saline) was administered by i.p. injection at a dose of 10µg/100g body weight 24h and 1h before decapitation. Control animals were injected with saline at a dose of 1ml/100g body weight 24h and 1h before decapitation.

5.2.2 Assays

Concentrations of CRH in plasma and hypothalamic tissue samples were determined as described in 2.3.6. Plasma samples of 25-50µl were extracted with 85% methanol and set up in single aliquots while standards and control pool samples were set up in duplicate. The lower limit of sensitivity ranged from 2-4pg per tube. Tissue samples were homogenised in 200µl 2M acetic acid and diluted 1:100 in 2M acetic acid, 100µl aliquots were evaporated to dryness,

reconstituted in assay buffer and assayed as described.

Oxytocin concentrations were determined as described in 2.6. Plasma samples of 10µl were assayed in single aliquots and standards in duplicate. The lower limit of sensitivity ranged from 4-6pg per tube.

Vasopressin concentrations were determined as described in 2.7. Plasma samples of 10µl and standards were assayed in duplicate. The lower limit of sensitivity ranged from 1-2pg per tube. Pituitary and hypothalamic tissue samples were homogenised in 100µl and 200µl 2M Acetic acid respectively and then diluted from 1:100 to 1:64000 in assay buffer. Aliquots of 100µl were assayed in duplicate.

The presence of AVP immunoreactivity in hypophysial portal blood was further examined by high performance liquid chromatography by Dr. I.C.A.F. Robinson as described in Robinson & Jones (1983). In brief, the portal blood sample was loaded onto a Nucleophil 5µm column with C_8 packing. The peptides were eluted at a flow rate of 1ml/min with a linear gradient from solvent A [0.1% v/v) TFA in water] to solvent B [(0.1% v/v) TFA in 60% acetonitrile] from 10-40% B over 30 min. The fractions collected were than analysed for AVP immunoreactivity by RIA as described in 2.6.

Concentrations of ACTH in plasma and anterior pituitary tissue samples were determined as described in 2.4. Plasma samples of 25-50µl were set up in duplicate and standards and pools in triplicate. The lower limit of sensitivity ranged from 5-10pg/ml.

Corticosterone concentrations were measured as described in 2.5. Single samples of peripheral plasma (20-50µl volume) were extracted with ether and aliquots of the extract dried down and set

up, with standards, in duplicate.

5.3 RESULTS

5.3.1 Peripheral plasma concentrations of ACTH and corticosterone

Trunk blood was collected from groups of six female Wistar, Long Evans homozygous and heterozygous Brattleboro rats. Figure 5.1 and 5.2 show that in the Wistar rat, concentrations of ACTH and corticosterone were significantly lower than in the Long Evans rat. There was no significant difference in the plasma ACTH and corticosterone concentrations between the homozygous Brattleboro and Long Evans rats but the concentrations of ACTH and corticosterone in the heterozygous Brattleboro rat were significantly lower than in the Long Evans (Fig. 5.1 and 5.2).

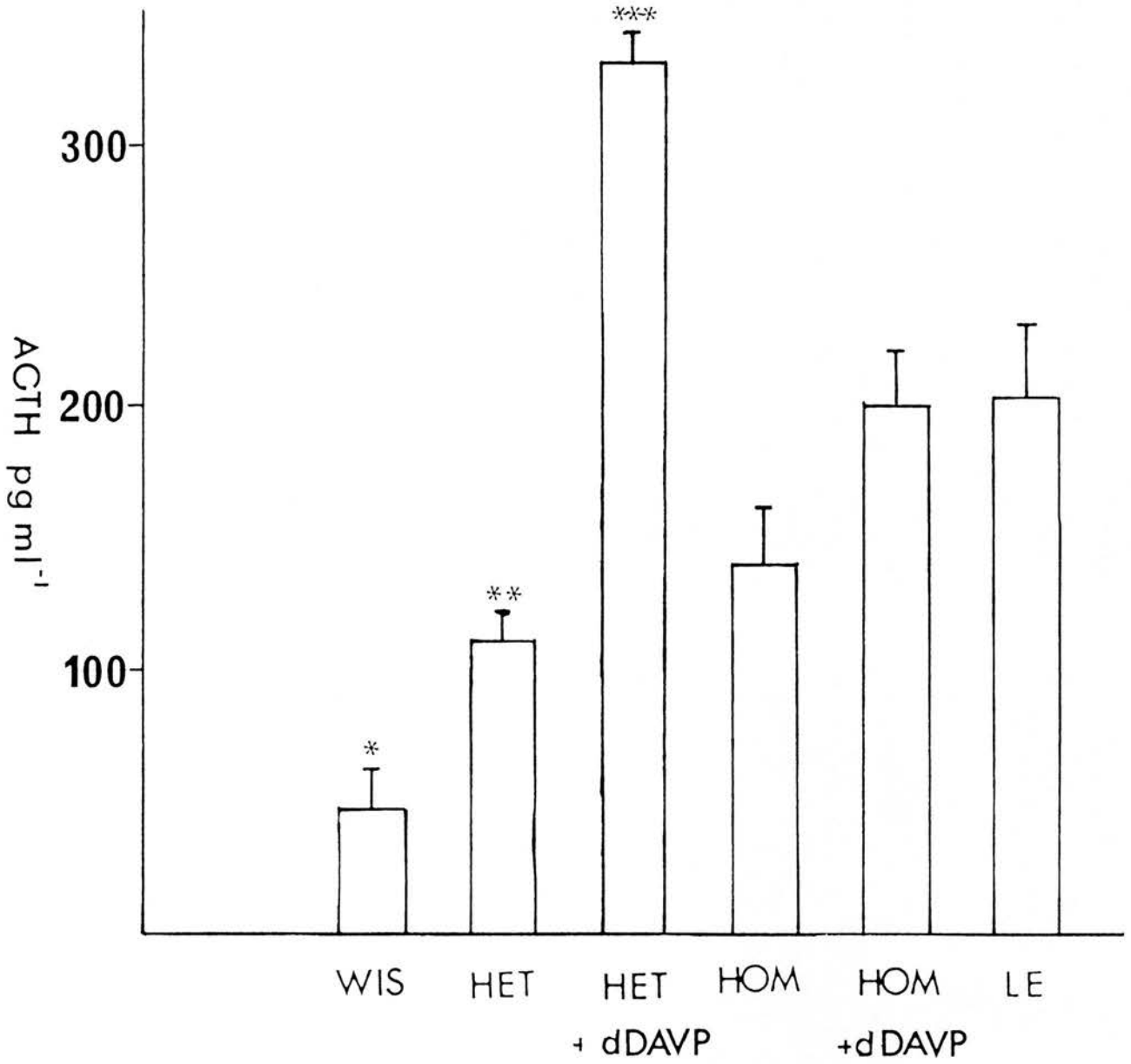
Injection of the vasopressin analogue, dDAVP, 24h and 1h before decapitation to heterozygous Brattleboro rats significantly increased ACTH and corticosterone concentrations 3- and 2-fold respectively, when compared with animals injected with saline alone (Fig. 5.1 and 5.2). In the homozygous Brattleboro rat, the increases in plasma ACTH and corticosterone concentrations after dDAVP injection were not significant when compared to homozygous rats injected with saline alone.

5.3.2 Concentrations of CRH, AVP and OT in hypophysial portal plasma

There was a significant difference in the concentration (Fig. 5.3) and content (Fig. 5.4) of CRH between the Wistar and the Long Evans rats. The concentration and content of CRH in the Long Evans rats was approximately 2-fold greater than in the Wistar

Figure 5.1 Peripheral plasma concentrations of ACTH in Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 6) concentration (pg ml^{-1}) of ACTH in peripheral plasma from female Wistar (WIS), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans (LE) rats. Rats were injected with either saline or dDAVP (+ dDAVP) 24h and 1h before decapitation. *$p < 0.005$, **$p < 0.02$ when compared with Long Evans rats. ***$p < 0.001$ when compared with saline treated heterozygous Brattleboro rats (Student's t-test).


ACTH pg ml-1
300
200
100
*
**
***
WIS
HET
HET + dDAVP
HOM
HOM +d DAVP
LE

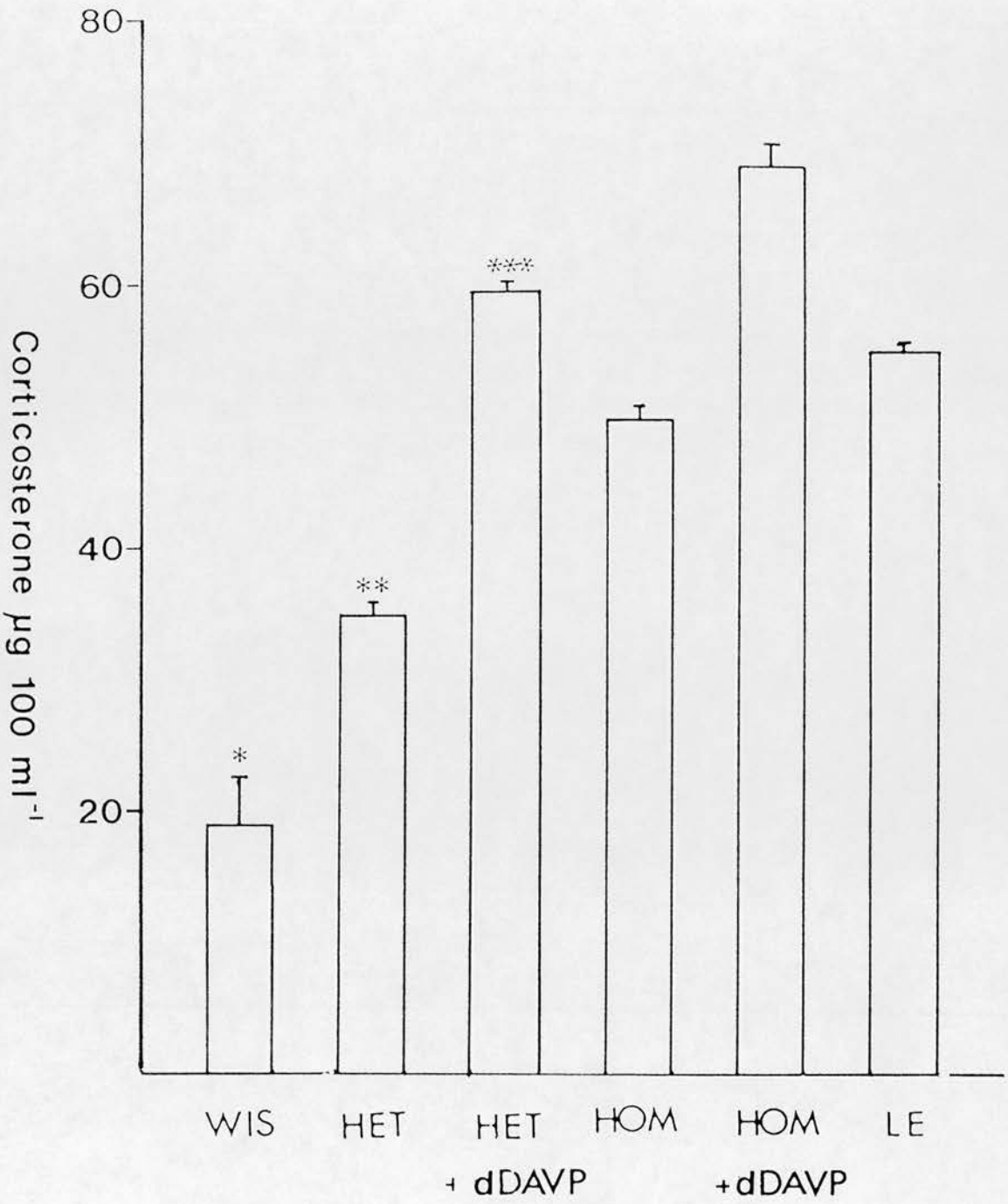


Figure 5.2 Peripheral plasma concentrations of corticosterone in Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 6) concentration (µg $100ml^{-1}$) of corticosterone in peripheral plasma from female Wistar (WIS), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans (LE) rats. Rats were injected with either saline or dDAVP (+ dDAVP) 24h and 1h before decapitation. *$p < 0.001$, **$p < 0.05$ when compared with Long Evans rats. ***$p < 0.01$ when compared with the saline treated Brattleboro rats (Student's t-test).

Figure 5.3 Concentration of CRH in hypophysial portal plasma of Wistar, Long Evans and Brattleboro rats.

Mean (± S.E.M.; n = 8) concentration of CRH (pg ml^{-1}) in hypophysial portal plasma from female Wistar (WIST), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans rats (LE). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2). *$p < 0.02$ when compared to Long Evans rats (Student's t-test).

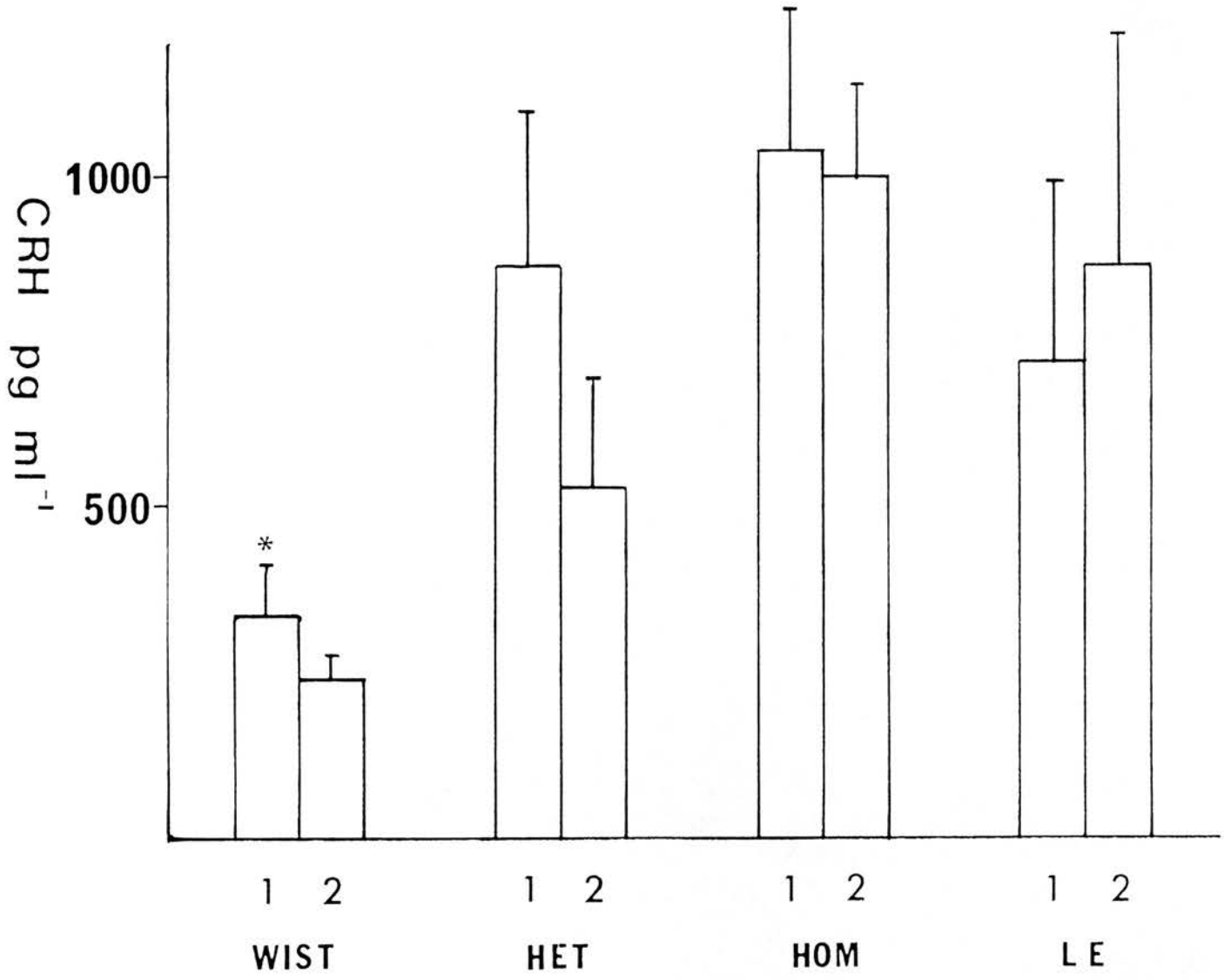
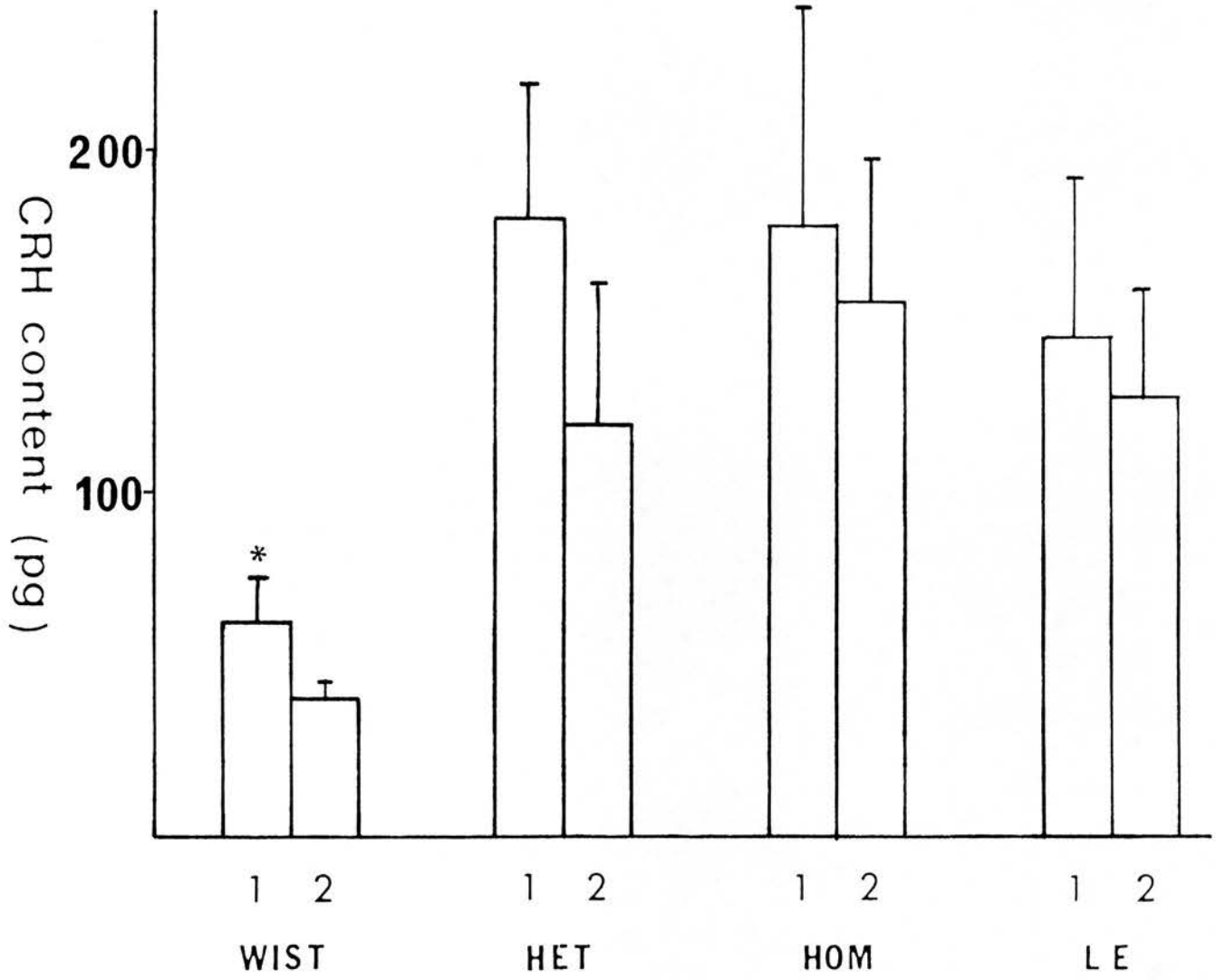

CRH pg ml-1
1000
500
*
1 2
WIST
1 2
HET
1 2
HOM
1 2
L E

Figure 5.4 Content of CRH in hypophysial portal plasma of Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 8) content of CRH (pg/30 min) in hypophysial portal plasma from female Wistar (WIST), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans rats (LE). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2). *$p < 0.02$ when compared with Long Evans rats (Student's t-test).


CRH content (pg)
200
100
*
1 2
WIST
1 2
HET
1 2
HOM
1 2
LE

rats. The concentrations and contents of CRH in the Long Evans control rats and homozygous or heterozygous Brattleboro rat were not significantly different (Fig. 5.3 and 5.4). The hypothalamic content of CRH in the Long Evans rat (31.8 ± 4.6ng/hypothalamus) did not differ significantly from the CRH content in the heterozygous Brattleboro (32.8 ± 2.4ng/hypothalamus) or in the homozygous Brattleboro rats (31.0 ± 4.0ng/hypothalamus).

Figures 5.5 and 5.6 show that there were no differences in the concentrations and contents of AVP in portal blood between the Wistar and Long Evans rats. There were no significant differences in the AVP concentrations or contents between the Long Evans control and heterozygous Brattleboro rats. AVP was consistently found in hypophysial portal blood from homozygous Brattleboro rats, in three separate experiments and using two different assay systems, at approximately 50% of the concentration and content found in Long Evans rats. The posterior pituitary content of AVP in the Long Evans rat was 582 ± 117ng/lobe and this was significantly higher than the heterozygous Brattleboro rat, 12 ± 2ng AVP/posterior lobe ($p < 0.001$; $n = 6$). AVP was undetectable in the posterior pituitary lobe and hypothalamus of the homozygous Brattleboro rat and hypothalamic AVP was detectable only in the Long Evans rats (21.6 ± 3.4ng/hypothalamus). AVP was undetectable in the anterior pituitary gland of Long Evans rats and was present in very low amounts in the heterozygous Brattleboro (0.1 ± 0.05ng/anterior pituitary) and in the homozygous Brattleboro rat (0.3 ± 0.1ng/anterior pituitary).

There was no significant difference in OT concentration (Fig. 5.7) or content (5.8) between Long Evans and Brattleboro rats or

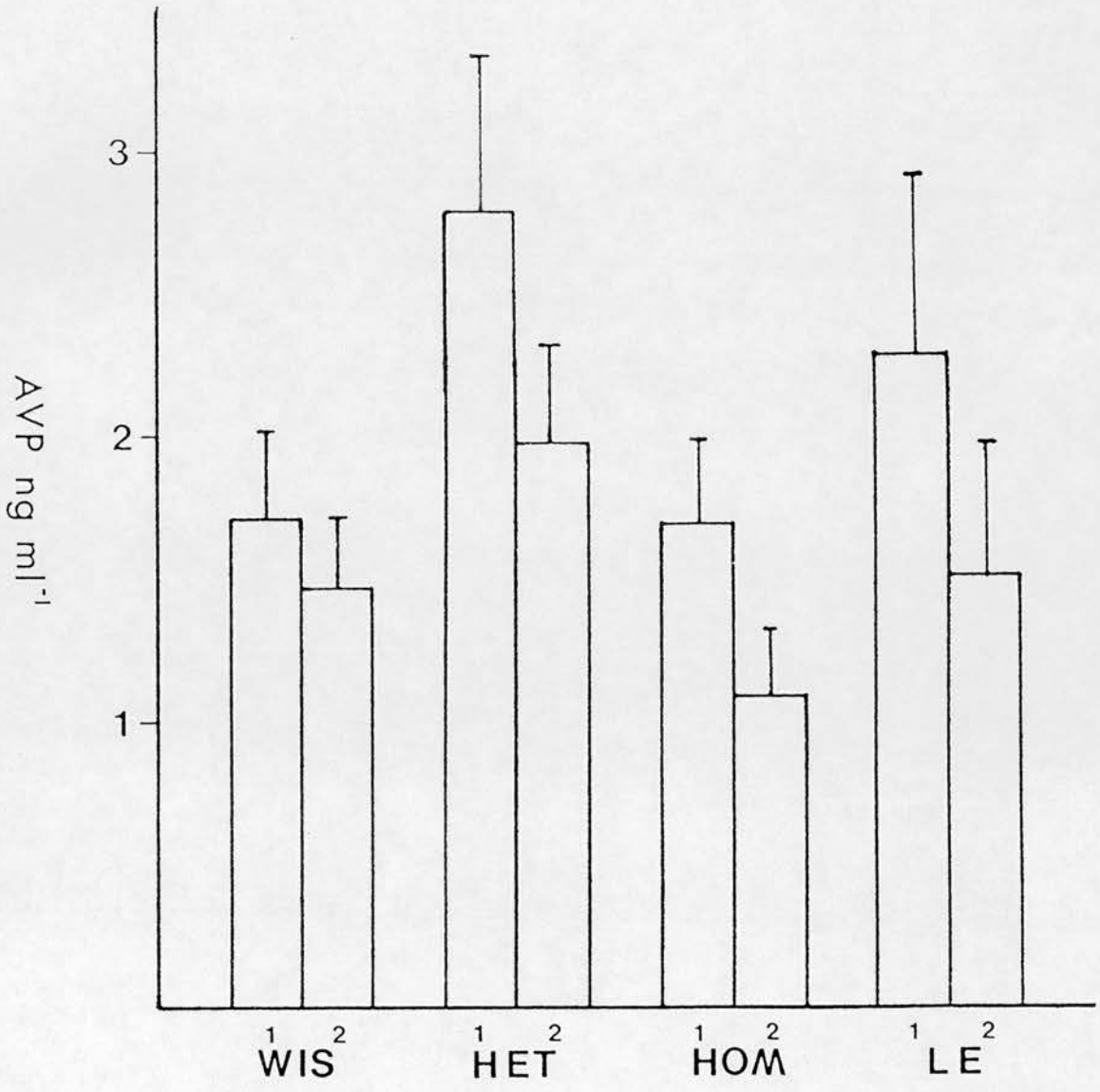


Figure 5.5 Concentration of Vasopressin (AVP) in hypophysial portal plasma of Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 8) concentration of AVP (ng ml^{-1}) in hypophysial portal plasma from female Wistar (WIS), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans rats (LE). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2).

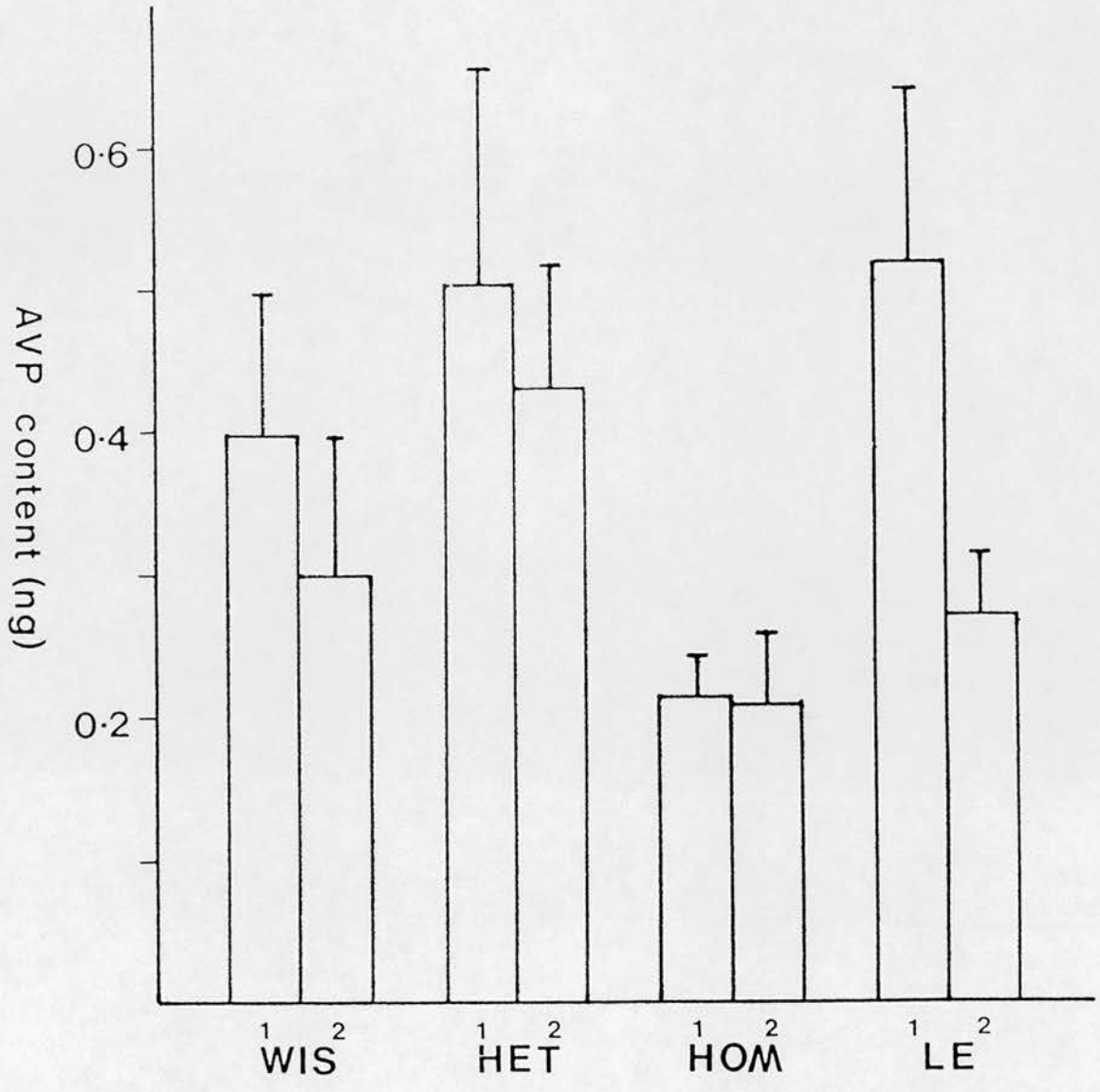


Figure 5.6 Content of Vasopressin (AVP) in hypophysial portal plasma of Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 8) content of AVP (ng/30 min) in hypophysial portal plasma from female Wistar (WIS), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans rats (LE). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2).

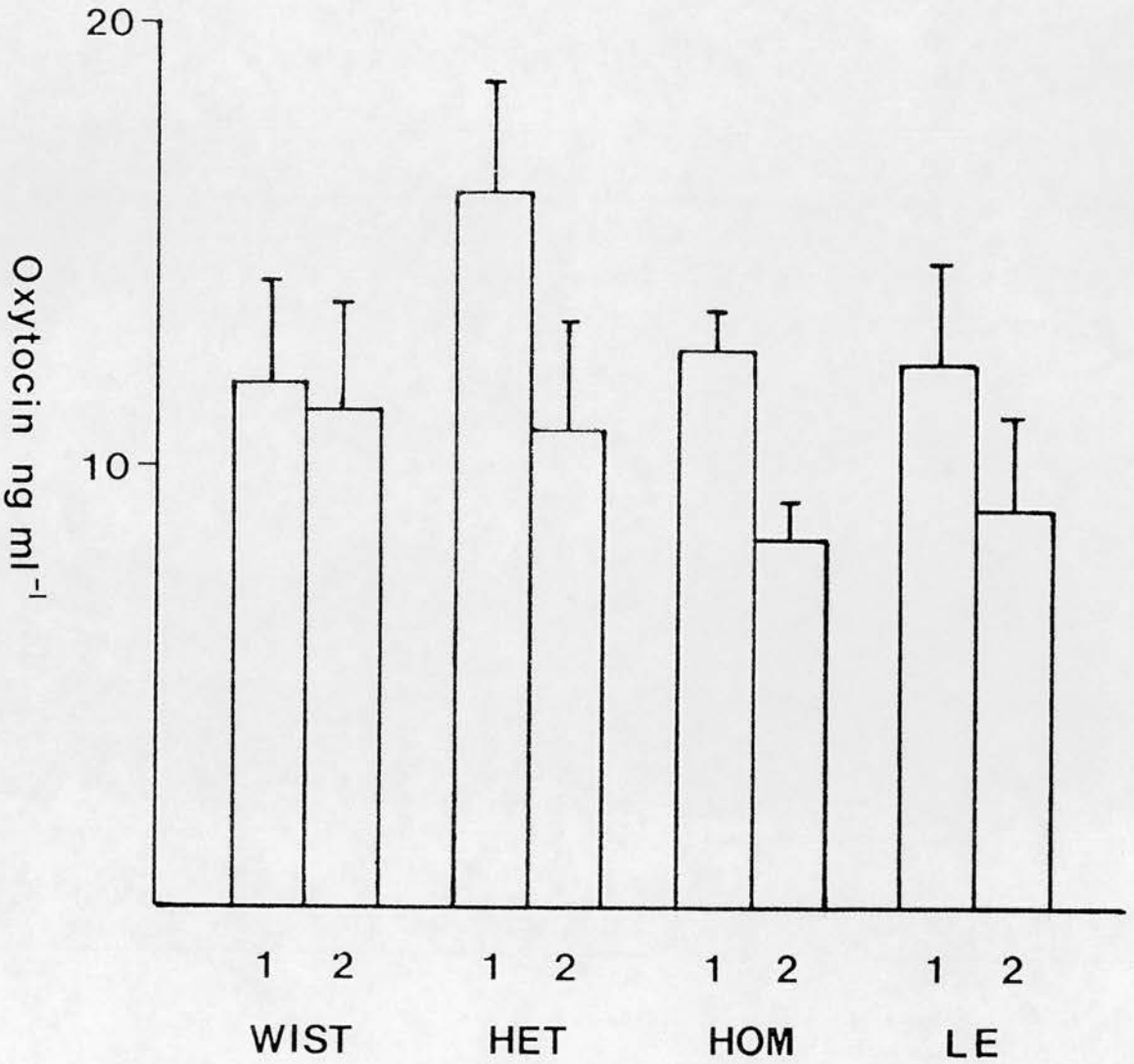


Figure 5.7 Concentration of Oxytocin (OT) in hypophysial portal plasma of Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 8) concentration of OT (ng ml^{-1}) in hypophysial portal plasma from female Wistar (WIS), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans rats (LE). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2).

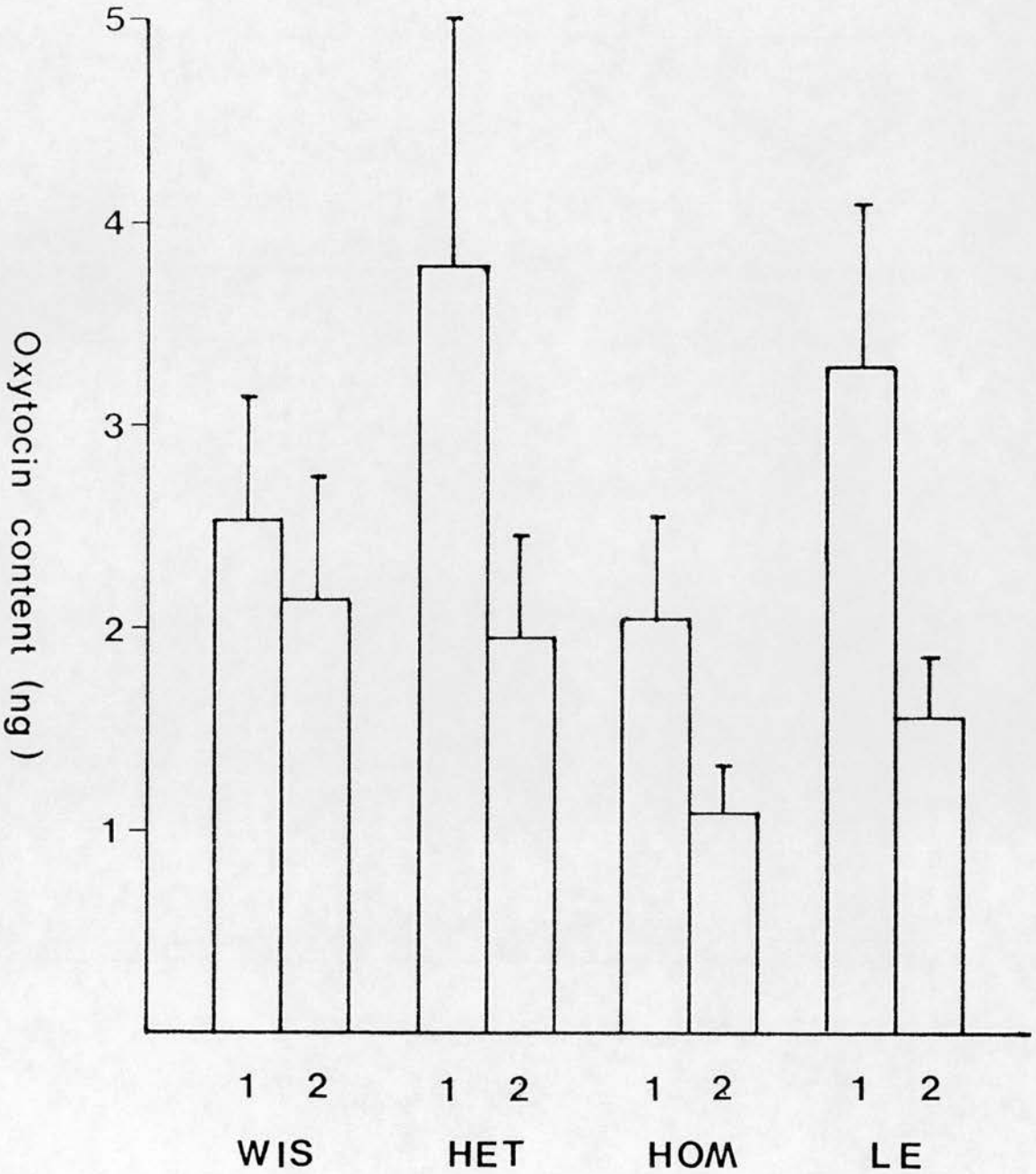


Figure 5.8 Content of Oxytocin (OT) in hypophysial portal plasma of Wistar, Long Evans and Brattleboro rats

Mean (± S.E.M.; n = 8) content of OT (ng/30 min) in hypophysial portal plasma from female Wistar (WIS), heterozygous Brattleboro (HET), homozygous Brattleboro (HOM) and Long Evans rats (LE). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2).

between Wistar and Long Evans rats.

Hypophysial portal blood extracts from homozygous Brattleboro rats and from Long Evans rats were examined by high performance liquid chromatography by Dr. I.C.A.F. Robinson. The AVP immunoreactivity from homozygous Brattleboro rats co-eluted with authentic from the Long Evans rats. The amount of AVP in the Brattleboro rat portal blood extract was approximately 33% of the amount found in the extract from Long Evans rats (Fig. 5.9).

5.4 DISCUSSION

These results show that there is a strain difference between Wistar and Long Evans rats in peripheral plasma concentrations of ACTH and corticosterone and that this difference can be attributed to a significantly lower concentration of CRH in portal blood in Wistar rats. The strain differences found between Long Evans and Wistar rats in ACTH and corticosterone concentrations demonstrates the importance of using the isogenic control for the Brattleboro rat. Surprisingly, the homozygous Brattleboro rat was repeatedly found to have vasopressin present in portal blood at concentrations of about 50% of those found in heterozygous rats and control Long Evans rats. There was no significant difference in CRH or OT concentrations in portal blood between either Brattleboro or Long Evans rats. ACTH and corticosterone concentrations in peripheral blood were lower in Brattleboro rats compared with the concentration in Long Evans rats but the difference was significant only in the heterozygous rat. Injection of dDAVP to homozygous and heterozygous Brattleboro rats increased ACTH and corticosterone secretion dramatically in the

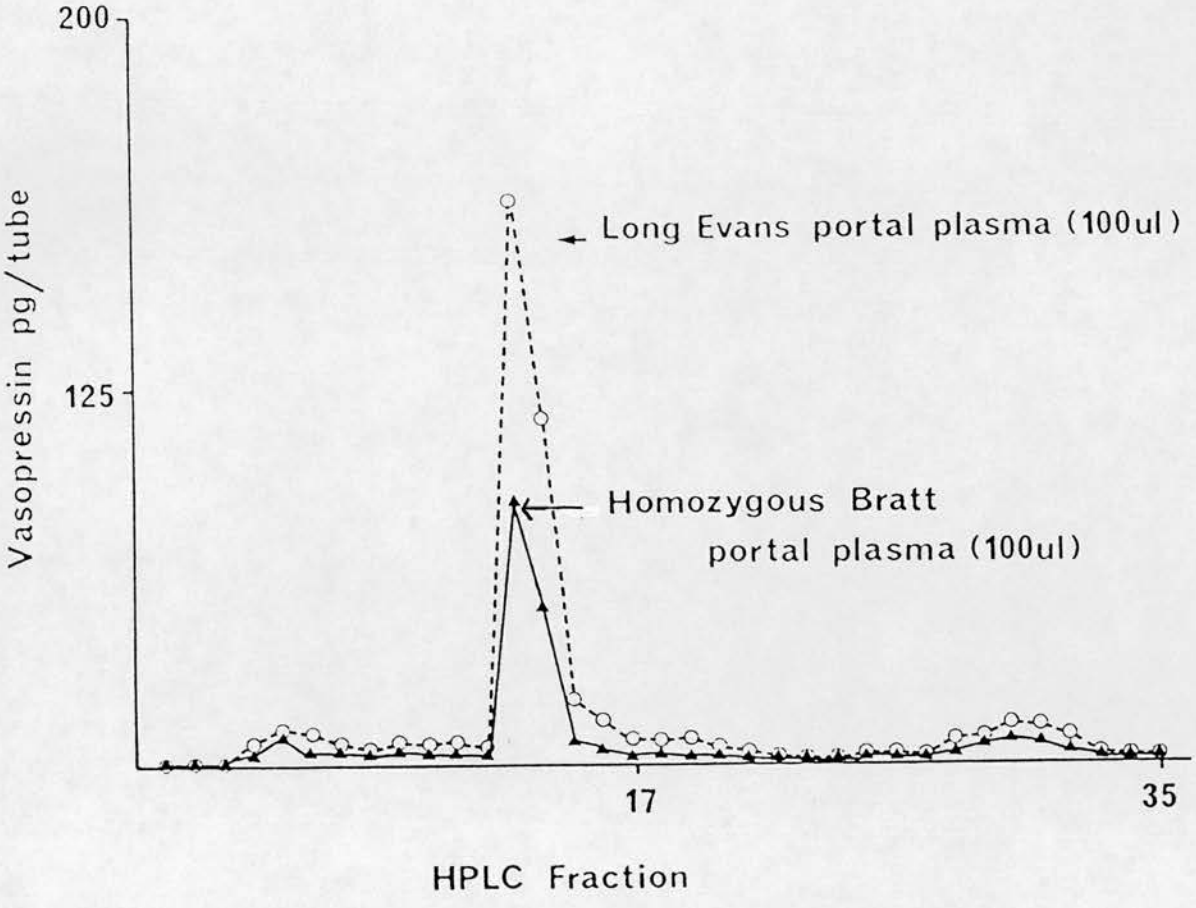


Figure 5.9 HPLC analysis of hypophysial portal plasma from Long Evans and Brattleboro rats

Hypophysial portal plasma was collected from Long Evans and homozygous Brattleboro rats and analysed by HPLC. Thirty-five 1ml fractions were collected and assayed for AVP by RIA. Results are expressed as vasopressin pg/tube.

heterozygous Brattleboro rat.

Immunoreactive AVP was found present in portal blood of homozygous Brattleboro rats. Classification of Brattleboro rats as homozygous was based on their urine output in a 24h period was equal to or greater than 75% of their body weight and by the absence of immunoreactive AVP in the posterior lobe of the pituitary gland. Chromatographically, the immunoreactive AVP in portal blood was identical to authentic AVP from Long Evans rats. The presence of AVP in portal blood in homozygous Brattleboro rats is not in agreement with Horn et al. (1985) who found AVP was undetectable in portal blood in homozygous Brattleboro rats. Although in the present study, AVP was not detectable in hypothalamic extracts from Brattleboro rats, the dilution factor was too great to measure the low amounts of AVP reported by other groups. Many groups have reported low concentrations of AVP in the hypothalamus of the homozygous Brattleboro rat (Lolait et al., 1986; Brownstein et al., 1980; Russell et al., 1980) thus providing a source for the AVP found in the portal blood from homozygous Brattleboro rats. Higher hypothalamic content of AVP has been shown in Long Evans rats (this study and Lolait et al., 1986) and this is reflected in the higher content of AVP in portal blood and in the posterior lobe of the pituitary gland. The heterozygous Brattleboro portal blood content of AVP was not significantly different from the Long Evans though hypothalamic and posterior pituitary content of AVP were substantially lower in the heterozygous Brattleboro rat.

The presence of AVP in hypophysial portal blood from homozygous Brattleboro rats is supported by the work of Mezey et al. (1986) who have shown AVP and C-terminal propressophysin immunoreactivity in

the PVN, supraoptic nuclei and in the posterior pituitary of homozygous Brattleboro rats. From these results, Mezey et al. (1986) suggest that homozygous Brattleboro rats make small amounts of the normal AVP precursor. The results of the present study and also Mezey's results are, at first sight, difficult to reconcile with the results of Schmale & Richter (1984), who have shown a genetic deficit in the Brattleboro rat which leads to an alteration in the AVP precursor. The presence of AVP in portal blood from homozygous Brattleboro rats suggests that (1) that the faulty AVP precursor is extracellularly processed after release; this would require the processing enzymes to work rapidly and efficiently to process the AVP after release and before collection of portal blood and is, therefore, unlikely (2) that the defect in the Brattleboro rats in the present study is not the same as the defect reported by Schmale & Richter (1984) or (3) the defect reported by Schmale & Richter (1984) would result in an alteration in the sequence of the C-terminus of the AVP precursor. It is possible that in the ovary, the adrenal gland and also in parvocellular neurones, the C-terminal component of the AVP precursor is not required for the processing of AVP, thereby explaining the tissue-specific distribution of AVP in the Brattleboro rat. However, the presence of C-terminal immunoreactivity in the adrenal gland, the testis and in the supraoptic and PVN of homozygous Brattleboro rats (Mezey et al., 1986) suggests this is not the case or (4) that Brattleboro and normal rats have more than one AVP gene. There is preliminary evidence to support the latter hypothesis as there appear to be more than two rat genes which hybridise to a neurophysin probe (Mezey et al., 1986). Processing of the different AVP genes in a tissue

specific manner could result in the high concentration of AVP in portal blood but very little AVP in the posterior pituitary gland.

Hypothalamic content of CRH and portal blood concentrations of CRH and OT were not significantly different in the three groups. ACTH and corticosterone concentrations in the homozygous Brattleboro rats were not significantly lower than control animals.

It would perhaps be expected, given the synergistic relationship between AVP and CRH in stimulating ACTH release, that such a reduced secretion of AVP would result in a much greater deficit in ACTH release. Results in chapter 3 indicate that in both genotypes of the Brattleboro rat, the mRNA encoding for the ACTH precursor (POMC) is 2-fold more abundant than in control Long Evans rats. It is, therefore, possible that the adrenocortical feedback system has a different set point in Brattleboro rats, the reduced concentration of corticosterone having less feedback effect on the anterior pituitary. Thus, in relation to Long Evans rats, POMC mRNA synthesis and ACTH release is increased in Brattleboro rats.

Laulin et al. (1985) have postulated that ACTH and corticosterone concentrations are higher than expected in Brattleboro rats due to the stress of water deprivation. In the present study, replacement of AVP with the synthetic analogue dDAVP in Brattleboro rats significantly increased the secretion of ACTH and corticosterone, but had no effect on POMC specific mRNA levels (Chapter 3). The present data is in agreement with Aizawa et al. (1981) who found ACTH secretion increased after injection with

dDAVP. The site of action of dDAVP appears to be on the pituitary since dDAVP also stimulates the secretion of ACTH from the isolated anterior pituitary (Pearlmutter et al., 1974). However, there is also a report by Andersson et al. (1972) that dDAVP, at doses much lower than used in this study, did not stimulate ACTH secretion in healthy male human subjects. In homozygous Brattleboro rats, administration of dDAVP has been shown to enhance hypothalamic staining for CRH (Burlet et al., 1983). Therefore, it is possible that dDAVP acts at the level of the hypothalamus to increase CRH release and also directly on the anterior pituitary gland to increase ACTH secretion without having an effect on POMC gene expression.

In conclusion, the homozygous Brattleboro rat has been shown to have AVP present in hypophysial portal blood at concentrations of approximately 50% of those found in Long Evans rats. The reduction in AVP in portal blood does not appear to affect ACTH release. The presence of AVP in portal blood in Brattleboro rats and the absence of AVP from the posterior pituitary gland suggests that (1) parvocellular neurones do not require the C-terminal component of the AVP precursor for the correct formation of AVP or (2) there may be more than one AVP gene, tissue specific processing of the different genes could result in AVP in portal blood being derived from a different AVP precursor to the precursor that supplies the neurones that project to the posterior pituitary gland.

CHAPTER 6

EFFECT OF ELECTRICAL STIMULATION OF THE MEDIAN EMINENCE ON VASOPRESSIN AND OXYTOCIN RELEASE INTO HYPOPHYSIAL PORTAL BLOOD

6.1 INTRODUCTION

For many years, AVP and OT were thought to be located exclusively in the posterior pituitary gland with projections coming from magnocellular neurones in the hypothalamus. More recently, immunohistochemical evidence has shown an OT- and AVP-containing pathway in mammals in the external layer of the median eminence with endings on the capillaries of the hypophysial portal system (Zimmerman, 1977). This pathway has also been demonstrated in birds using Gomori staining (Oksche et al., 1974). Further studies have shown that the origin of this AVP and OT is in parvocellular neurones of the PVN (Swanson et al., 1983; Merchanthaler et al., 1983), suggesting that there are two distinct systems involved in the secretion OT and AVP. One system which originates in parvocellular neurones, terminates in the external layer of the median eminence and releases OT and AVP into portal blood and another system which originates in the magnocellular neurones and terminates with the release of OT and AVP from the posterior pituitary gland. AVP can be released from median eminence *in vitro* (Beny & Baertschi, 1981) and release can be increased by stimulation with depolarising concentrations of potassium (Holmes et al., 1986). Both AVP and OT have been found in high concentrations in hypophysial portal blood at concentrations approximately 10- to 20-fold greater than the values in peripheral blood (Zimmerman et al., 1973; Recht et al., 1981; Gibbs, 1984; Horn et al., 1985).

Electrical stimulation of the median eminence *in vivo* increased AVP and OT release into peripheral blood but not into hypophysial portal blood (Horn et al., 1985). The possible explanations

offered by Horn et al. (1985) were that the frequency of stimulation used may have been too high or that the neurones were fatigued by the frequency or by the length of pulse trains. However, both these explanations were unlikely as the same frequency and length of stimulation caused a significant release of AVP and OT into peripheral blood in intact animals. The third, and most likely explanation, was that exposure and subsequent cutting of the stalk somehow leads to maximal activity of the VP and OT neurones so that release of these peptides cannot be increased by stimulation. The study by Horn et al. in 1985 was carried out under urethane anaesthesia which has been shown, in Chapter 4, to substantially elevate ACTH and corticosterone concentrations. The choice of anaesthesia may account for the fact that Horn et al. (1985) found that electrical stimulation of the median eminence did not increase AVP and OT release into portal blood although other explanations cannot be excluded. Endogenous opioids may regulate OT and AVP release from the posterior pituitary. Opioid peptides are present both within (Martin & Voight, 1981; Weber & Barchas, 1983; Zamir et al., 1985) and around (Van Leeuwen et al., 1983) magnocellular neurones in the supraoptic and paraventricular nuclei that synthesise AVP or OT. Metenkephalin has been found to be co-localised with oxytocin and dynorphin and related peptides co-localised with vasopressin. In addition, opiate receptors are present in the neural lobe of the pituitary and in the supraoptic and paraventricular nuclei of the hypothalamus of the monkey (Womsley et al., 1982). In the isolated neurohypophysis, reports on the effect of opioid agonists and antagonists on electrically stimulated AVP release have been variable with both inhibition of

AVP release by opioid agonists (Iversen et al., 1980; Lightman et al., 1982) and no effect of opioid agonists (Bicknell et al., 1985b) being reported. Data on the effect of opioid antagonists on electrical stimulation of AVP from the neurohypophysis are also conflicting. Maysinger et al. (1984) and Knepel & Meyer (1983) have reported that naloxone increases the electrically stimulated release of AVP from the neurohypophysis while Bicknell & Leng (1982) and Bicknell et al. (1985a) found no effect of opioid antagonists on stimulated AVP release. The inhibitory effect of endogenous opioid peptides on electrically stimulated OT release from the neurohypophysis *in vitro* has been demonstrated by the work of many groups, showing that naloxone infusion markedly increased the secretion of OT in response to electrical stimulation of the neurohypophysis (Clarke et al., 1979; Bicknell & Leng, 1982; Maysinger et al., 1984).

These data demonstrate that an opioid ligand is released within the posterior pituitary gland together with AVP and OT and acts to inhibit OT and possibly AVP secretion. The aims of the present study were to examine the effect of anaesthesia on release of AVP and OT and to establish whether endogenous opioid peptides inhibit OT and AVP release into hypophysial portal blood during electrical stimulation of the median eminence.

6.2 MATERIALS AND METHODS

6.2.1 Animals and Surgery

Female COB Wistar rats (200-250g body weight), purchased from Charles River U.K. Ltd. (Margate, Kent), were maintained under

controlled lighting (lights on 0500-1900h) and temperature (22°C) and allowed free access to diet 41B (Oxoid, Basingstoke, Hants) and tap water.

Anaesthesia was induced with either urethane (ethyl carbamate 1g kg^{-1} body weight) or sagatal (sodium pentobarbitone 36mg kg^{-1} body weight). One group of animals was anaesthetised with urethane 20 min after anaesthesia was induced and given an i.v. loading dose of 1mg naloxone hydrochloride (Sigma Chemicals) in 0.5ml 0.9% saline via the external jugular vein 1h before blood collection. Circulating concentrations of naloxone were kept constant during blood collection by infusion of naloxone 1.55mg ml^{-1} at a rate of 0.54ml h^{-1} into the external jugular vein. A control group of animals received an infusion of saline instead of naloxone.

The method of collection of hypophysial portal blood and electrical stimulation of the median eminence were as described in 2.2.6 and 2.2.7. Hypophysial portal blood was collected into chilled plastic tubes containing Trasylol 1000 KIU /ml blood. Blood was collected during two consecutive 30 min periods with electrical stimulation applied to the median eminence of some animals during the second collection period. The electrical stimulus was generated by a Neurolog constant current stimulator (Digitimer, Welwyn Garden City, Herts) and a stimulus of biphasic rectangular waves with parameters of frequency 50Hz, pulse width 1ms, amplitude of 1mA, was applied in trains of 30 sec on and 30 sec off.

6.2.2 Assays

Oxytocin and Vasopressin concentrations in portal and peripheral

plasma were determined as described in 2.6. Plasma samples of 10µl were set up in single aliquots and standards in duplicate. The lower limit of sensitivity for oxytocin ranged from 4-6pg/tube and for vasopressin from 2-3pg/tube.

6.3 RESULTS

6.3.1 Volume of hypophysial portal plasma collected

Table 1 shows that the volumes of hypophysial portal plasma collected in the first 30 min in the groups infused continuously either with saline or naloxone, were significantly greater than the volumes collected in the groups not infused with saline. The volume of plasma collected during stimulation of the median eminence in the non-infused group was significantly greater than the volume collected before application of the stimulus. Because of these differences in volumes of portal plasma, the values of OT and AVP in portal plasma are expressed as content (concentrations x volume of plasma collected) as well as concentration.

6.3.2 Stimulation of OT and AVP into hypophysial portal blood

Hypophysial blood was collected under anaesthesia with Sagatal and stimulation was applied during the second 30 min collection period to one group. Peripheral blood samples were withdrawn from the external jugular vein immediately before sectioning of the stalk and at the end of collection of portal blood. Fig. 6.1 and 6.2 show respectively that there was no significant increase in either the concentration or content of AVP or OT during the second 30 min collection period with or without median eminence stimulation. In

TABLE 6.1

Mean (± S.E.M.) volumes (μl) of hypophysial portal plasma collected during two consecutive 30 min periods

Experimental group	Number of animals per group	0-30 min	30-60 min
Non-infused, control	8	211 ± 29	173 ± 21
Non-infused, stimulated	8	153 ± 13	198 ± 12*
Saline infused, stimulated	7	315 ± 57**	478 ± 98*
Naloxone infused, stimulated	7	376 ± 54**	477 ± 88

Adult female Wistar rats were anaesthetised with sagatal and hypophysial portal blood collected from them. Some groups were infused continuously with either saline or naloxone. Stimulation was applied to the median eminence during the second 30 min period. *$p < 0.05$ (paired student's t-test) when compared with volume of plasma collected during the first 30 min period. **$p < 0.02$ (student's t-test) when compared with plasma volume collected in the non-infused groups.

Figure 6.1 Vasopressin concentration and content in hypophysial portal blood before and during stimulation

Mean (± S.E.M.; n = 8) a) concentration (ng ml^{-1}) and b) content (ng/30 min) of vasopressin (AVP) in hypophysial portal plasma in female Wistar rats collected during two consecutive 30 min periods (1 and 2). Stimulation was applied to the median eminence during the second 30 min collection period (Stim), in control animals (Con) no stimulation was applied.

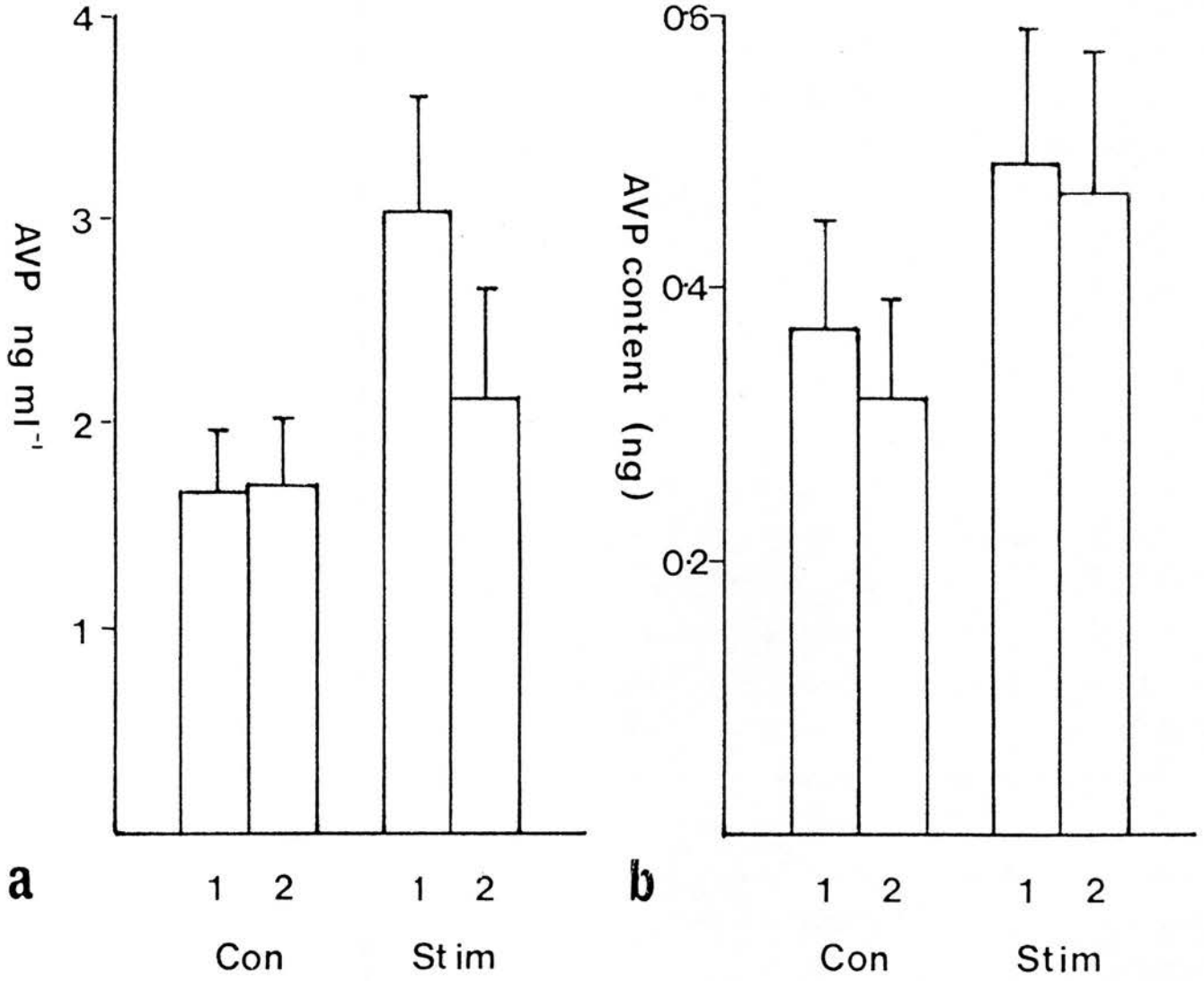
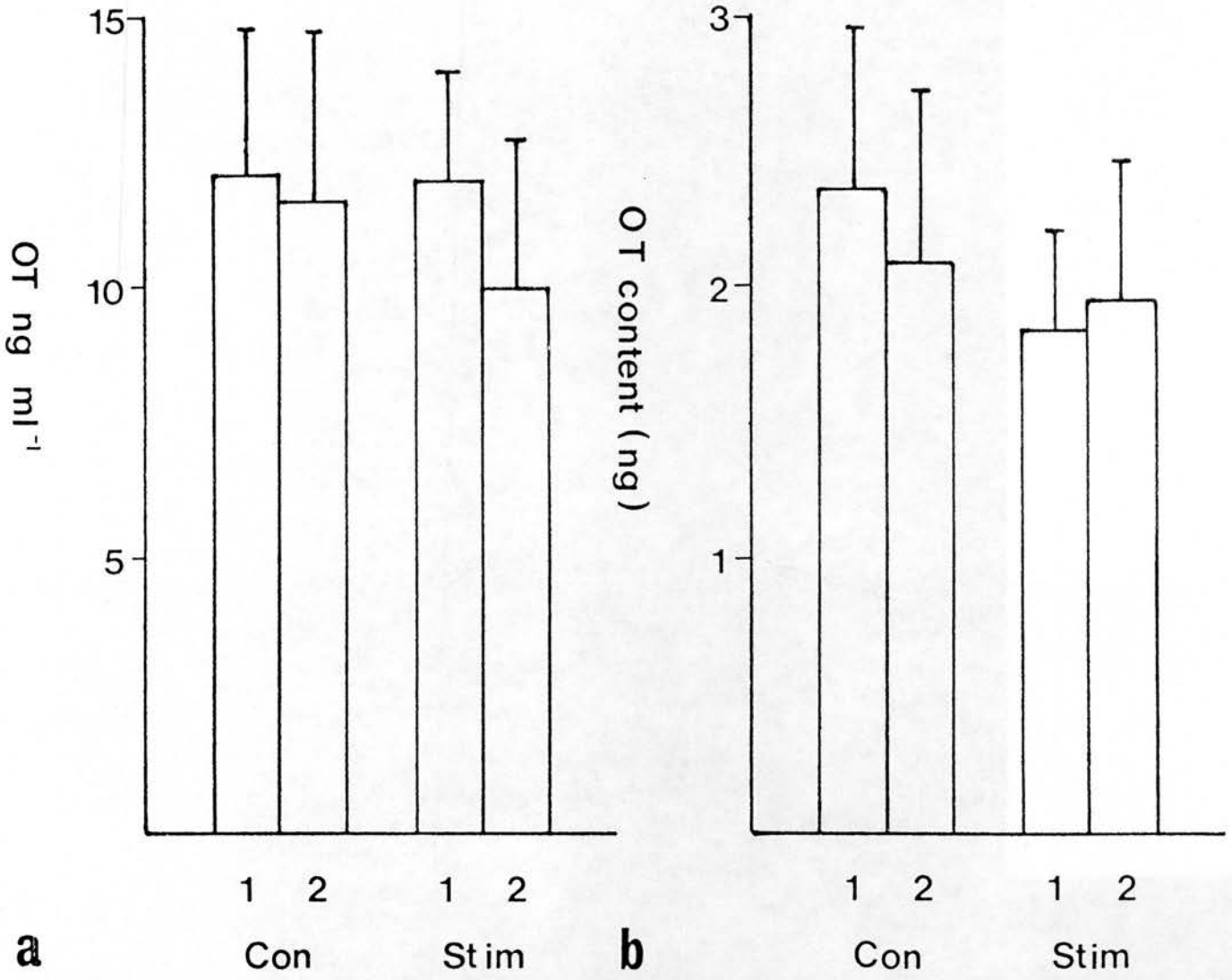

AVP ng ml⁻¹
4
3
2
1
a
1 2 1 2
Con Stim
AVP content (ng)
0·6
0·4
0·2
b
1 2 1 2
Con Stim

Figure 6.2 Oxytocin concentration and content in hypophysial portal blood before and during stimulation

Mean (± S.E.M.; n = 8) a) concentration ng ml^{-1}) and b) content (ng/30 min) of oxytocin (OT) in hypophysial portal plasma in female Wistar rats collected during two consecutive 30 min periods (1 and 2). Stimulation was applied to the median eminence during the second 30 min collection period (Stim), in control animals (Con) no stimulation was applied.


15
10
5
OT ng ml-1
1 2 1 2
Con Stim
a
3
2
1
OT content (ng)
1 2 1 2
Con Stim
b

* Peripheral plasma concentrations of OT and AVP

Control group; 0.25 ± 0.19 ng/ml OT, 0.17 ± 0.13 ng/ml AVP.

Stimulated group (prestimulation); 0.3 ± 0.15 ng/ml OT,
0.19 ± 0.16 ng/ml AVP

Saline infused group; 0.3 ± 0.1 ng/ml OT, 0.28 ± 0.19 ng/ml AVP

Naloxone infused group; 0.26 ± 0.15 ng/ml OT, 0.14 ± 0.12 ng/ml AVP

all the groups, peripheral plasma concentrations of OT and AVP* were significantly ($p < 0.001$) lower than these found in portal plasma.

Figs. 6.3 and 6.4. show that continual infusion with either saline or naloxone to animals anaesthetised with urethane did not significantly affect concentration or content of either AVP or OT in hypophysial portal plasma. In all the groups, peripheral plasma concentrations of AVP and OT* were significantly lower ($p < 0.001$) than portal plasma concentrations.

6.4 DISCUSSION

These results, which show that OT and AVP are present in hypophysial portal plasma in higher concentrations than in peripheral plasma, are in agreement with previous findings (Oliver et al., 1977; Recht et al., 1981; Gibbs, 1984; Horn et al., 1985). Stimulation of the median eminence did not increase release of OT or AVP into portal blood, as was the case in the study by Horn et al. (1985) under urethane anaesthesia. As shown in Chapter 4, urethane anaesthesia substantially elevates the plasma concentrations of ACTH and corticosterone. The lack of effect of median eminence stimulation on AVP and OT release into portal blood could be explained by the fact that the anaesthesia and pituitary stalk section could lead to maximum release of OT and AVP neurones so that stimulation could not further increase the release of these peptides into portal blood. However, this explanation for the lack of effect of median eminence stimulation on the release of AVP of maximum output cannot be reconciled easily with the significant increase in AVP found in adrenalectomised animals. In the present study, anaesthesia was induced with sagatal which does not elevate

Figure 6.3 Effect of naloxone on stimulated release of vasopressin into hypophysial portal blood

Mean (± S.E.M.; n = 7) a) concentration (ng ml^{-1}) and b) content (ng/30 min) of vasopressin (AVP) in hypophysial portal blood in female Wistar rats collected during two consecutive 30 min periods (1 and 2). Animals were infused continuously with either saline (Sal) or naloxone (Nal) and stimulation applied to the median eminence during the second 30 min period.

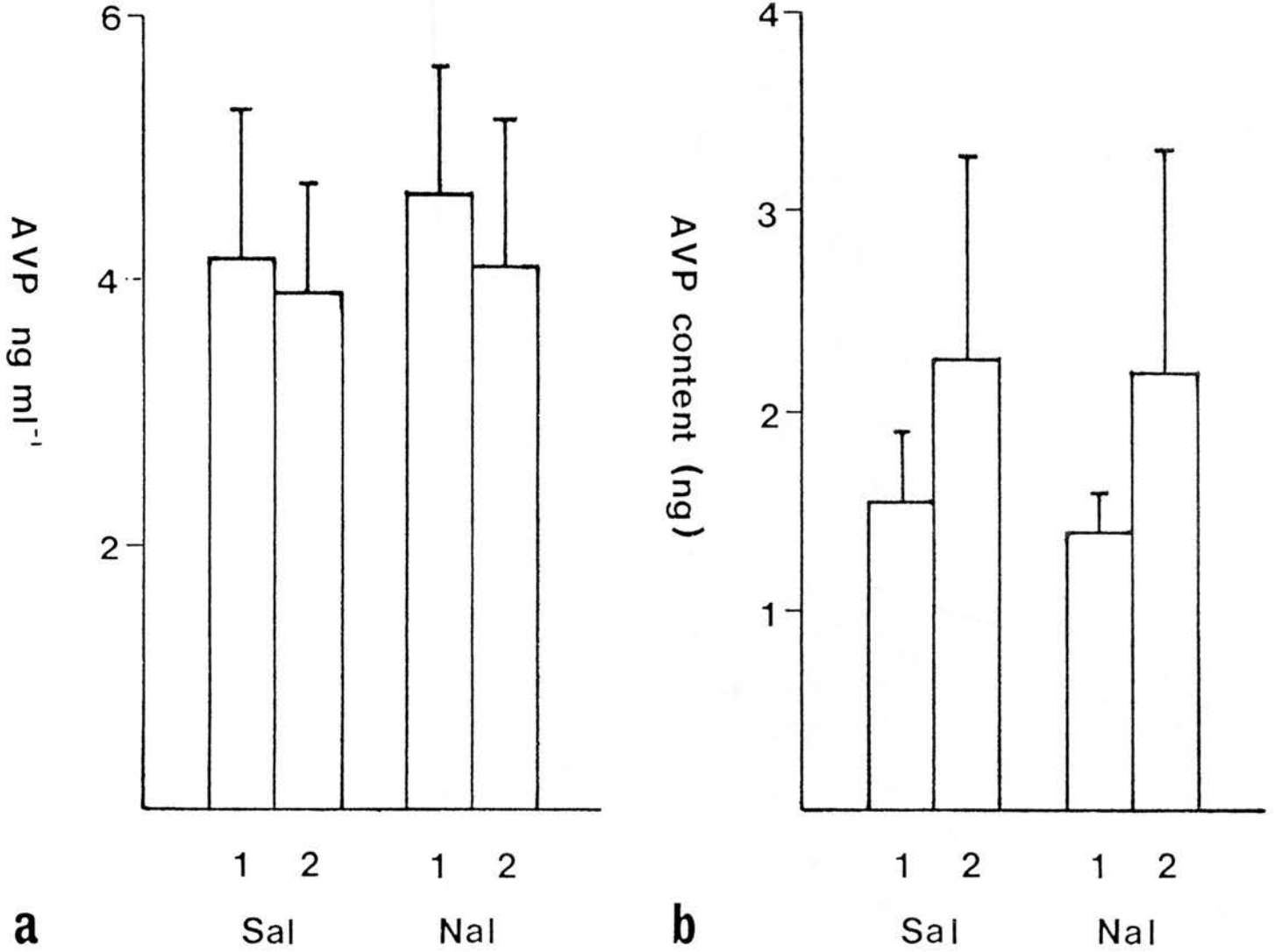
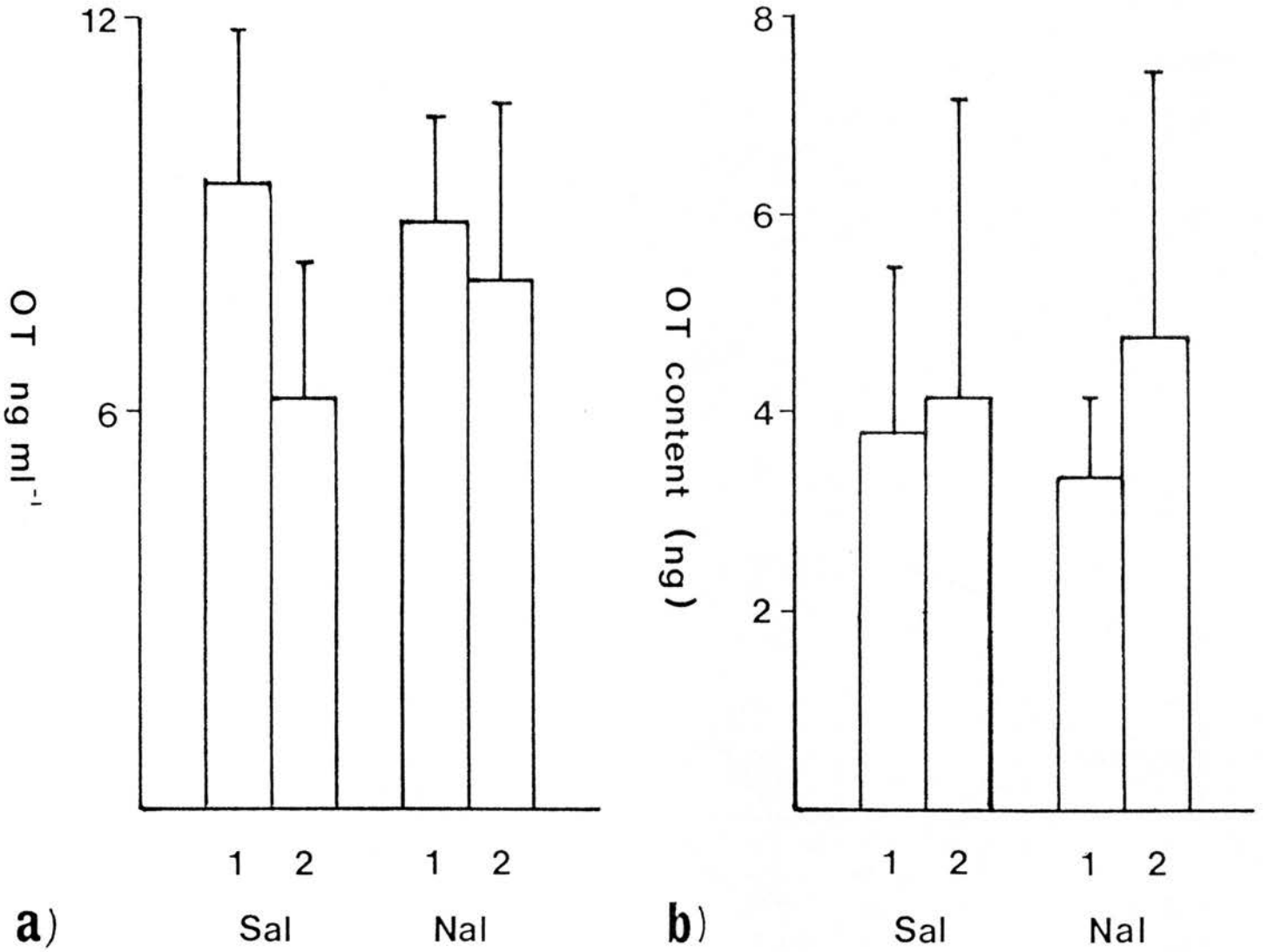

AVP ng ml⁻¹
6
4
2
1 2 1 2
Sal Nal
a
AVP content (ng)
4
3
2
1
1 2 1 2
Sal Nal
b

Figure 6.4 Effect of naloxone on stimulated release of oxytocin into hypophysial portal blood

Mean (± S.E.M.; n = 7) a) concentration (ng ml^{-1}) and b) content (ng/30 min) of oxytocin (OT) in hypophysial portal blood in female Wistar rats collected during two consecutive 30 min periods (1 and 2). Animals were infused continuously with either saline (Sal) or naloxone (Nal) and stimulation applied to the median eminence during the second 30 min period.


12
6
OT ng ml-1
1 2 1 2
Sal Nal
a)
8
6
4
2
OT content (ng)
1 2 1 2
Sal Nal
b)

stress hormones so dramatically (Chapter 4) when compared with urethane anaesthesia. There was no increase in the release of AVP or OT on stimulation of the median eminence with either anaesthetic. In the present study, comparison between pre-stimulation amounts of AVP and OT released under urethane and sagatal is difficult due to the infusion of saline to the rats anaesthetised with urethane. It is, therefore, not possible to ascertain whether urethane anaesthesia increased the release of AVP and OT into portal blood to a greater extent than anaesthesia with sagatal.

The frequency of the electrical stimulus used has produced an increased release of other peptides into hypophysial portal blood (Fink & Jamieson, 1976; Sheward et al., 1984) and has also increased release of AVP and OT into peripheral blood (Horn et al., 1985). Endorphins are synthesised in the hypothalamus (Smyth, 1983) and are released into hypophysial portal blood (Wardlaw et al., 1982; Sakar & Yen, 1985). Therefore, the possibility that stimulated release of AVP and OT is inhibited by opioid peptides was examined by blockade of opioid receptors with naloxone. Stimulation of the median eminence under infusion with naloxone failed to increase the release of AVP and OT into portal blood. Although there are conflicting views regarding the _in vitro_ effects of naloxone on AVP secretion from the posterior pituitary, with both facilitation and lack of effect being reported (Bicknell & Leng, 1982; Maysinger et al., 1984; Bicknell et al., 1985a), the stimulatory action of naloxone and k-antagonists on _in vivo_ and _in vitro_ OT secretion from the posterior pituitary has been well established (Bicknell & Leng, 1982; Bicknell et al., 1984;

Bicknell et al., 1985a). Therefore, it is surprising that naloxone infusion had no effect in the present study on basal or stimulated release of AVP and OT. β-endorphin has been shown to have a dual effect on CRF release from the rat hypothalamus *in vitro* with a stimulatory response at low concentrations and an inhibitory effect at higher concentrations (Buckingham, 1986). The stimulatory effect of β-endorphin on CRF secretion is readily antagonised by naloxone and has been shown to be mediated by μ opioid receptors while the inhibitory action of β-endorphin on CRF release is only antagonised by concentrations of naloxone high enough to interact with δ opioid receptors (Buckingham & Cooper, 1986). Opioid substances normally produce an effect by inhibiting the release of neurotransmitters and, therefore, the inhibitory action of β-endorphin may be exerted on δ receptors on the releasing-factor secreting neurones and on different Λ receptors at the pituitary level. The different receptor types may explain the difference in the effect of naloxone at the hypothalamic and pituitary level. The concentrations of naloxone used in the present study may have been insufficient to block δ receptors and, therefore, the lack of effect of naloxone cannot rule out the possibility that opioid peptides prevent an increased release of AVP and OT into hypophysial portal blood in response to electrical stimulation.

CHAPTER 7

EFFECT OF ELECTRICAL STIMULATION OF THE BRAIN ON THE HYPOTHALAMO-PITUITARY ADRENOCORTICAL SYSTEM

7.1 INTRODUCTION

Central neural control of adrenocortical function has been the subject of numerous studies. However, there is still controversy regarding the role of various brain structures in the control of pituitary-adrenal function. The neural apparatus which regulates adrenocortical function is generally held to have two components; a medial basal hypothalamic component which maintains a basal level of secretory activity and a limbic-midbrain component which is involved in the circadian rhythm and stress induced ACTH release (Yates & Maran, 1975).

The involvement of the hypothalamus in control of adrenocortical function is well established. The parvocellular region of the paraventricular nuclei (PVN) of the hypothalamus contains AVP and CRH neurones that terminate on hypophysial portal vessels in the median eminence. CRH and AVP are transported to the anterior pituitary where they stimulate ACTH release, which in turn stimulates release of corticosterone from the adrenal cortex (for details see Chapter 1). Glucocorticoid receptor density is high in the hypothalamus, particularly in the PVN where there is considerable overlap between cells containing a high concentration of glucocorticoid receptors and cells containing immunoreactive CRH (Fuxe et al., 1985). Stimulation of the lateral hypothalamus resulted in a marked increase in plasma cortisol in the monkey (Frankel et al., 1978) and stimulation of the PVN increased the ACTH concentration in plasma of cats (Dornhorst et al., 1981). The increase in plasma concentrations of ACTH and corticosterone in response to hypothalamic stimulation has been suggested to be due to stimulated release of CRH and/or AVP from the median eminence. CRH

and AVP release from the isolated median eminence can be stimulated by depolarising concentrations of potassium (Holmes et al., 1986). However, electrical stimulation of the median eminence or PVN did not increase the release of AVP from the median eminence into hypophysial portal blood (Horn et al., 1985). The lack of effect of electrical stimulation of the median eminence in releasing AVP is surprising given that the frequency used for stimulation had resulted in a significant release of AVP and OT into peripheral blood in intact animals. Horn et al. (1985) concluded that exposure and subsequent cutting of the pituitary stalk may lead to maximum activity of the AVP neurones so that release of the peptide could not be further increased by stimulation.

Species differences as well as differences in methodology may account for some discrepancies in changes in plasma corticosterone following stimulation or lesioning of the amygdala. Increased adrenocortical activity has been reported to follow bilateral removal of the amygdaloid and pre-amygdaloid cortex of cats and dogs (Martin et al., 1958), electrolytic destruction of the amygdala of rats (Yamada & Greer, 1980) or ablation of the medial amygdala nuclei in deer mice (Eleftheriou et al., 1966). Decreased activity followed transection of the stria terminalis and bilateral amygdalectomy of concious monkeys (Mason, 1959). An increase in plasma corticosterone concentrations have been reported to follow electrical stimulation of the amygdala of conscious cats where pre-stimulation concentrations of corticosterone were low. In the cat, when corticosterone concentrations were elevated due to anaesthesia with urethane, stimulation of the corticomedial amygdala caused inhibition of corticosterone release suggesting that the

inhibitory effect of stimulation of the amygdala is masked in animals with low prestimulation concentrations of corticosterone (Matheson et al., 1971). However, plasma corticosterone concentrations also increased after stimulation of the amygdala in conscious rats (Carrillo & Dunn, 1977) and in rats anaesthetised with pentobarbitone (Redgate & Fahringer, 1973). The various individual nuclei of the amygdala project differently onto the hypothalamus (Krettek & Price, 1978) and in the anaesthetised rat, Dunn & Whitener (1986) have shown that stimulation of the different amygdaloid nuclei have different effects on plasma concentrations of corticosterone; stimulation of the central and lateral amygdaloid nuclei produced a decrease in plasma corticosterone concentrations and stimulation of the basomedial, medial and posterior corticomedial nuclei resulted in increased plasma corticosterone concentrations.

The hippocampus has been shown to exert mainly an inhibitory effect in the regulation of ACTH secretion (Casady & Taylor, 1976; Mangili et al., 1966) and also to play a role in maintaining the circadian rhythm of adrenocortical function (Moberg et al., 1971). The hippocampus is a main target region for corticosterone (McEwen, et al., 1979). Type 2 glucocorticoid receptors (as described in 1.4.1) are present in high concentrations in the CA1 and CA2 regions of the hippocampus (Fuxe et al., 1985). Detailed analysis of changes in plasma concentrations of corticosterone following stimulation of the hippocampus has shown that in the anaesthetised rat, specific changes can be related to distinct sites within the hippocampus. An increase in plasma corticosterone concentration was observed following stimulation of the CA1 region. In contrast,

stimulation of CA3, the dentate gyrus and the subiculum produced significant decreases in plasma corticosterone concentrations (Dunn & Orr, 1984).

The aim of the present study was to examine the effect of stimulation of the limbic and hypothalamic structures presumed to be involved in adrenocortical function on the release of CRH and AVP into hypophysial portal blood. The changes in CRH and AVP release into portal blood were correlated with the changes in plasma concentrations of ACTH and corticosterone after brain stimulation in rats in which the pituitary stalk was intact.

7.2 MATERIALS AND METHODS

7.2.1 Animals and Surgery

Adult female Wistar COB rats (200-250g body weight) purchased from Charles River U.K. Ltd. (Margate, Kent) were maintained under controlled lighting (lights on 0500h-1900h) and temperature (22°C) and allowed free access to diet 41B (Oxoid, Basingstoke, Herts) and tap water. Animals that were subjected to surgery and allowed to recover had access to drinking water to which had been added aureomycin (50mg l^{-1}).

The electrodes were implanted according to the co-ordinates of de Groot (1959) and were as described in section 2.2.8.

Animals were allowed to recover for 7-9 days before the collection of hypophysial portal blood. Median eminence stimulation was carried out as described in 2.2.7.

Hypophysial portal blood was collected under anaesthesia with sagatal over two consecutive 30 min periods as described in 2.2.6.

Stimulation was applied during the second 30 min period using a constant current generator and the stimulus was based on the parameters used by Jamieson & Fink (1976). Hypophysial portal blood was collected every minute and immediately mixed with Trasylol 1000KIU/ml blood in chilled plastic tubes. Peripheral blood samples were taken from the external jugular vein immediately before section of the pituitary stalk and mixed with Trasylol, 2000KIU/ml blood in chilled plastic tubes. All blood samples were spun for 20 min at 1,720 x g at 4°C and plasma stored at -40°C for assay.

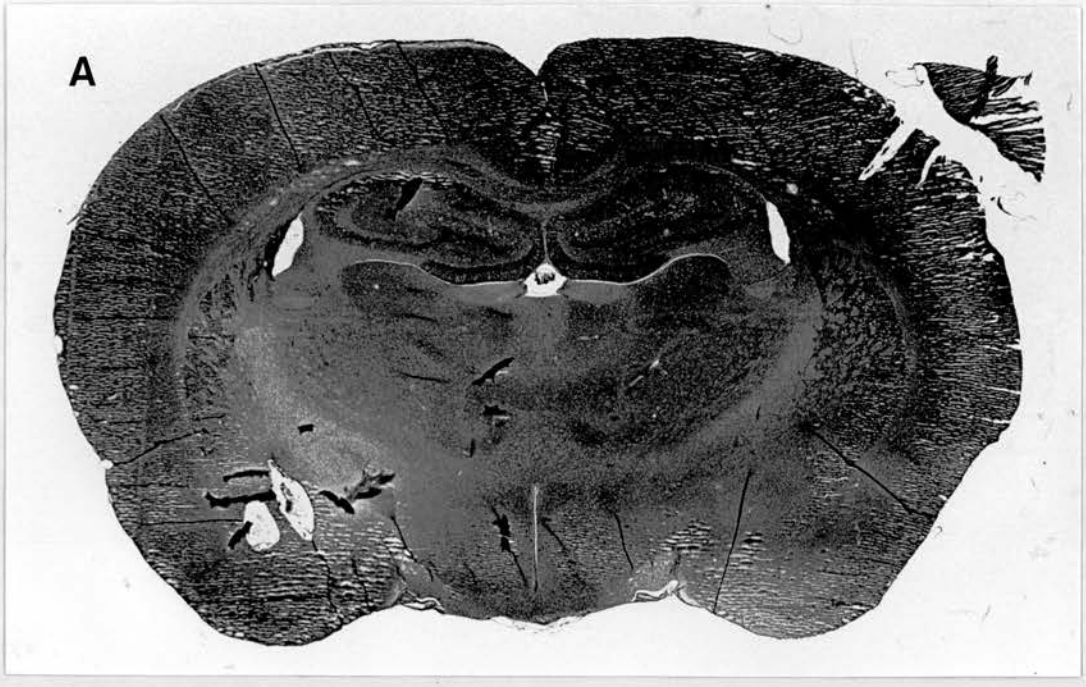
In some animals, electrodes were implanted and anaesthesia induced with sagatal. The external jugular vein was exposed and peripheral blood samples taken immediately before and after 30 min of stimulation as described above.

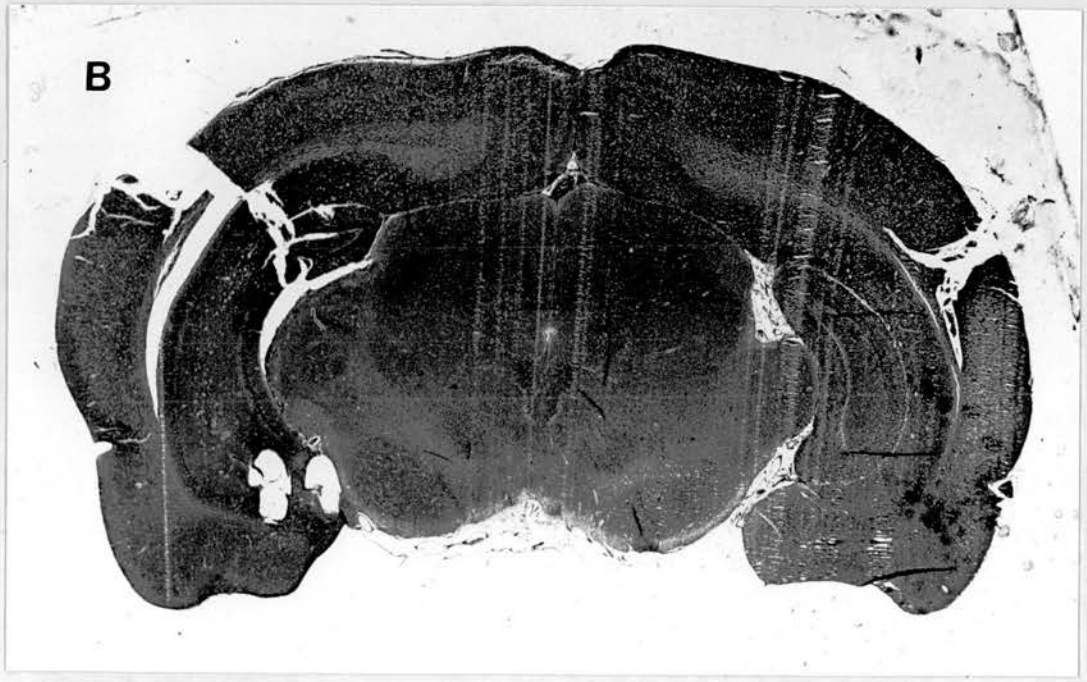
7.2.2 Localisation of site of stimulation

At the end of each experiment, animals were decapitated and the head placed in 4% formalin in 0.9% saline for seven days. For the removal of the electrodes, the skin was cut off from the area covering the electrodes and the dental cement. The dental cement was removed by placing the heads, dorsal surface downward and supported by a piece of aluminium foil, in a glass container filled with chloroform. After the cement had been dissolved, the electrodes were gently removed from the brain. The brain was then removed from the skull and stored in 4% formalin in 0.9% saline overnight. The tissue was embedded in paraffin wax and serial 10µm frozen sections of the brain were prepared and stained with thionine for microscopic assessment of the position in the brain of the stimulating electrodes. Fig. 7.1 shows representative sections

Figure 7.1 Positioning of stimulating electrodes in rat brain

Representative coronal sections of rat brain stained with thionine showing the position of the stimulating electrode tips in A) the amygdala (AMY) B) the ventral hippocampus (HIP) and C) the paraventricular nuclei (PVN).


A


B


C

with electrodes positioned in the amygdala, hippocampus and PVN.

7.2.3 Assays

Concentrations of CRH in plasma samples were determined as described in 2.3.6. Plasma samples of 50-100µl were extracted with 85% methanol and set up in single aliquots due to the small sample volume while standards and pools were extracted and set up in duplicate. The lower limit of sensitivity ranged from 2-4pg per tube.

Vasopressin concentrations were determined as described in 2.7. Plasma samples of 10µl and standards were set up in duplicate. The lower limit of sensitivity ranged from 1-2pg per tube.

Concentrations of ACTH in peripheral plasma samples were determined as described in 2.4. Plasma samples of 20µl were set up in duplicate with standards and pools in triplicate. The lower limit of sensitivity ranged from 5-10pg ml^{-1}.

Corticosterone concentrations were measured as described in 2.5. Single samples of 20µl of peripheral plasma were extracted with ether, aliquots of the extract dried down and set up in the assay in duplicate. Standards were made in duplicate in methanol, dried down and set up in the assay.

7.3 RESULTS

7.3.1 Volume of hypophysial portal plasma collected

The volumes of portal plasma collected during the two collection periods are shown in Table 7.1. Stimulation of most brain areas

TABLE 7.1

Mean (± S.E.M.) volumes (μl) of hypophysial portal plasma collected during two consecutive 30 min periods

Experimental group	Number of animals per group	0–30 min	30–60 min
AMY control	6	315 ± 55	298 ± 52
PVN stimulated	17	297 ± 51	591 ± 134*
ME stimulated	8	153 ± 13	198 ± 12*
AMY stimulated	6	248 ± 56	318 ± 79
HIP stimulated	8	190 ± 35	244 ± 44*

*$p < 0.05$ (paired student's t test) when compared with volume of plasma collected during the first 30 min collection period. Stimulation was applied during the second 30 min period. AMY - amygdaloid; PVN - paraventricular nuclei; ME - median eminence; HIP - hippocampus.

significantly increased the volume of plasma collected and, therefore, the results are expressed as content (concentration x volume of plasma) as well as concentration.

7.3.2 Stimulation of CRH and AVP into hypophysial portal blood

In 'control' animals in which electrodes had been implanted in the amygdala but no stimulus applied, there was no significant difference in content (Fig. 7.2) or concentration (Fig. 7.3) of CRH in portal plasma during the two collection periods. Stimulation of the median eminence during the second 30 min period increased the release of CRH into portal blood but not significantly. However, stimulation of the amygdala significantly decreased the content (Fig. 7.2) of CRH and stimulation of the hippocampus significantly decreased the concentration (Fig. 7.3) of CRH in portal blood by 40-50%. The CRH response to PVN stimulation depended on the position of the tips of the stimulating electrodes. Individual values of CRH content before and during stimulation are shown in Table 7.2. Figure 7.4 shows that in animals in which the electrode tips were within 0.2mm of the PVN, stimulation produced a significant release of CRH into portal blood so that CRH content increased 3-fold relative to that in the 30 min period before the stimulus was applied (Fig. 7.2). Figures 7.5. and 7.6 show that neither content or concentration of AVP in portal blood was significantly affected by stimulation of the various brain areas.

7.3.3 Stimulation of ACTH and corticosterone into peripheral blood

Wistar rats that had been implanted with an electrode were anaesthetised with sagatal and peripheral blood samples taken from

Figure 7.2 Effect of electrical stimulation on CRH content in hypophysial portal plasma

Mean (± S.E.M.) content (pg/30 min) of corticotrophin-releasing hormone (CRH) in hypophysial portal plasma from female Wistar rats with electrodes implanted in the amygdala (AMY; n = 6), hippocampus (HIP; n = 8), median eminence (M.E.; n = 8) and paraventricular nuclei* (PVN; n = 8). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2) with electrical stimulation applied during the second 30 min collection period. Control rats (CON; n = 6) had electrodes implanted in the amygdala, but no stimulation was applied. *p < 0.02 compared with the control (no stimulation) collection period (paired t-test).

* only sites of stimulation close to the PVN have been included.

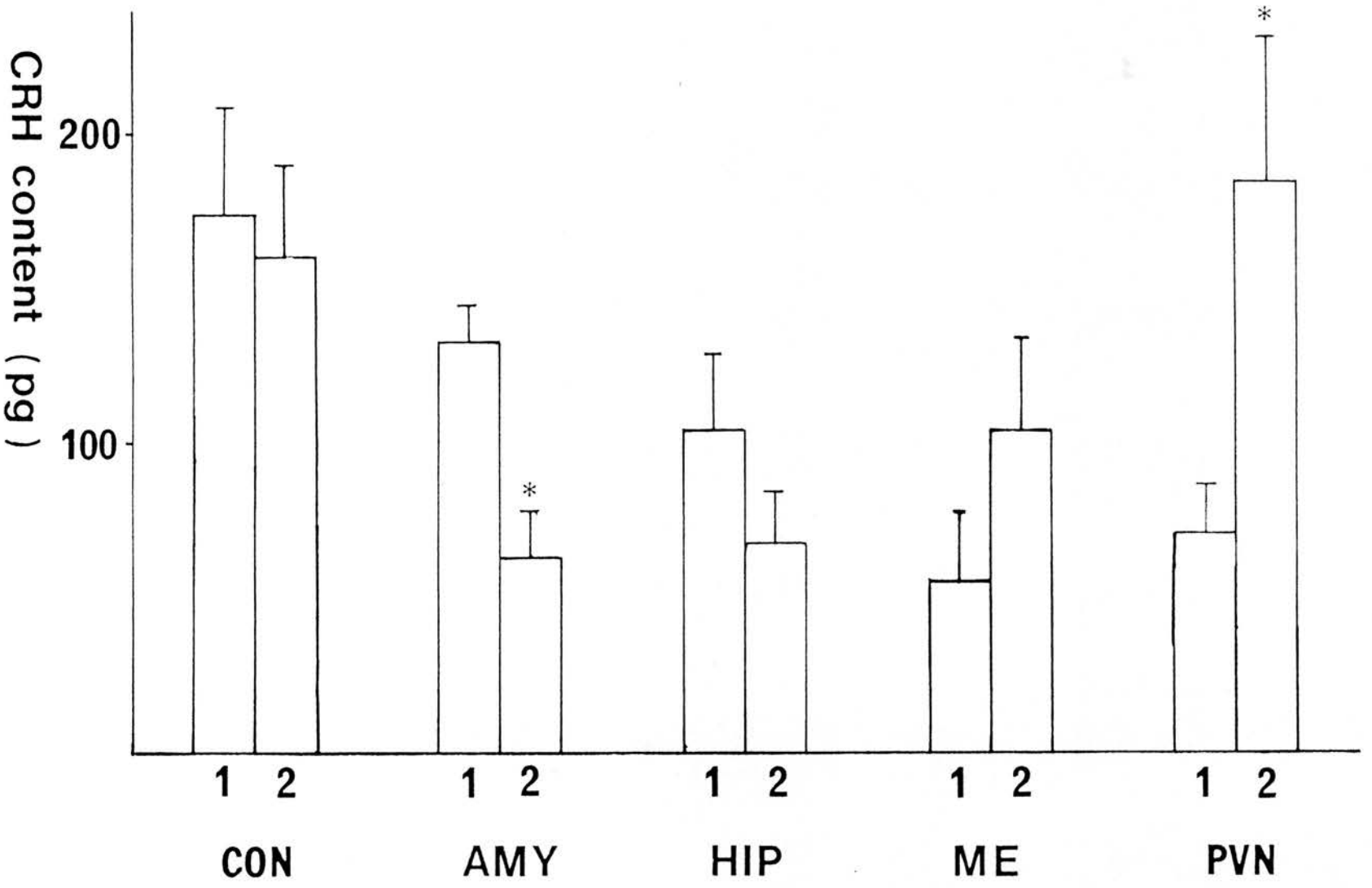

CRH content (pg)
200
100
*
*
1 2
1 2
1 2
1 2
1 2
CON
AMY
HIP
ME
PVN

Figure 7.3 Effect of electrical stimulation on CRH concentration in hypophysial portal plasma

Mean (± S.E.M.) concentration (pg ml^{-1}) of corticotrophin-releasing hormone (CRH) in hypophysial portal plasma from female Wistar rats with electrodes implanted in the amygdala (AMY; n = 6), hippocampus (HIP; n = 8), median eminence (M.E.; n = 8) and paraventricular nuclei* (PVN; n = 8). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2) with electrical stimulation applied during the second 30 min collection period. Control rats (CON; n = 6) had electrodes implanted in the amygdala, but no stimulation was applied. *p < 0.01 compared with the control (no stimulation) collection period (paired t-test).

* only sites of stimulation close to the PVN have been included.

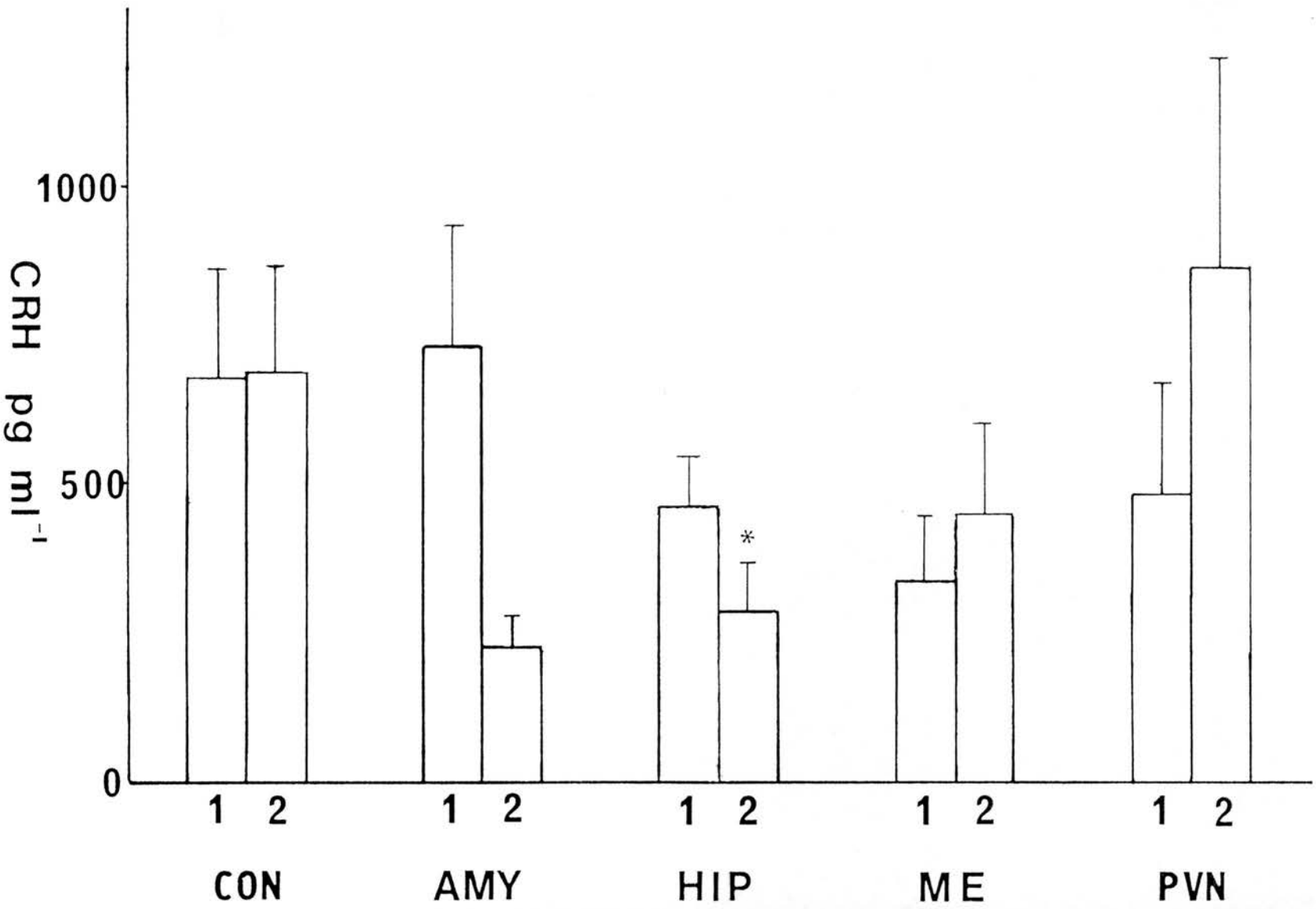

1000
500
0
CRH pg ml-1
*
1 2
1 2
1 2
1 2
1 2
CON
AMY
HIP
ME
PVN

TABLE 7.2

Content (pg/30 min) of CRH in hypophysial portal plasma of individual female Wistar rats with stimulating electrodes implanted in the PVN. Hypophysial portal blood was collected during two consecutive 30 min periods with electrical stimulation applied during the second 30 min period

Animal number	CRH content (pg/30 min)	
	0–30 min	Stimulation
1	88	240
2	42	312
3	83	74
4	65	45
5	172	411
6	188	189
7	126	45
8	58	195
9	38	90
10	75	76
11	95	88
12	64	74
13	74	53
14	60	105
15	26	36
16	208	53

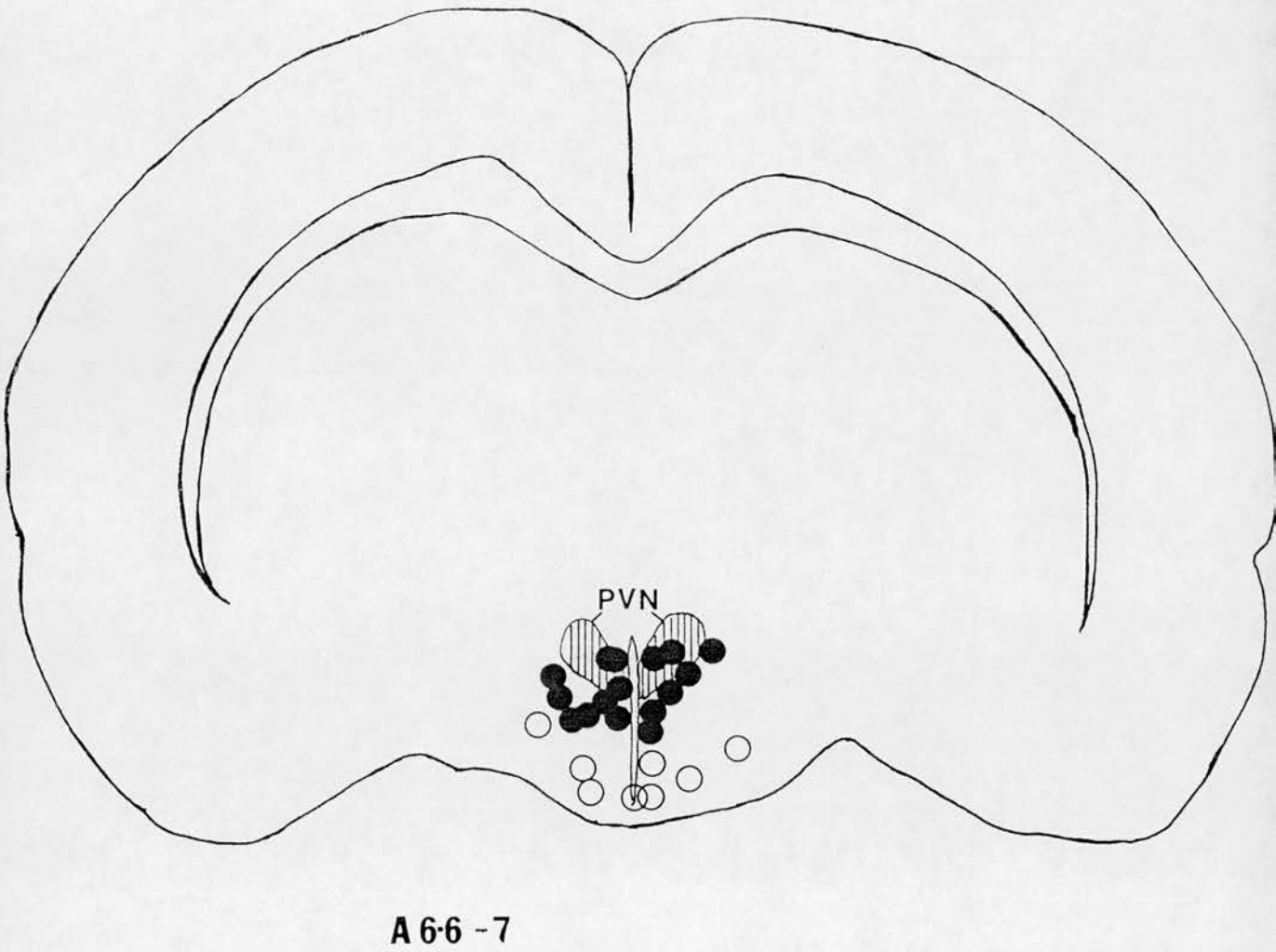


Figure 7.4 Positioning of stimulating electrodes in the PVN and their effectiveness in stimulating CRH release

Schematic coronal section of rat brain redrawn from de Groot (1959), illustrating the distribution of stimulation sites around the paraventricular nuclei (PVN). Stimulation of sites marked (●) increased release of CRH into hypophysial portal plasma and stimulation of sites marked (○) decreased release of CRH into portal plasma when compared with the control, non-stimulated release of CRH into portal plasma.

Figure 7.5 Effect of electrical stimulation on AVP content in hypophysial portal plasma

Mean (± S.E.M.) content (ng/30 min) of vasopressin (AVP) in hypophysial portal plasma from female Wistar rats with electrodes implanted in the amygdala (AMY; n = 6), hippocampus (HIP; n = 8), median eminence (M.E.; n = 8) and paraventricular nuclei (PVN; n = 8). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2) with electrical stimulation applied during the second 30 min collection period. Control rats (CON; n = 6) had electrodes implanted in the amygdala, but no stimulation was applied.

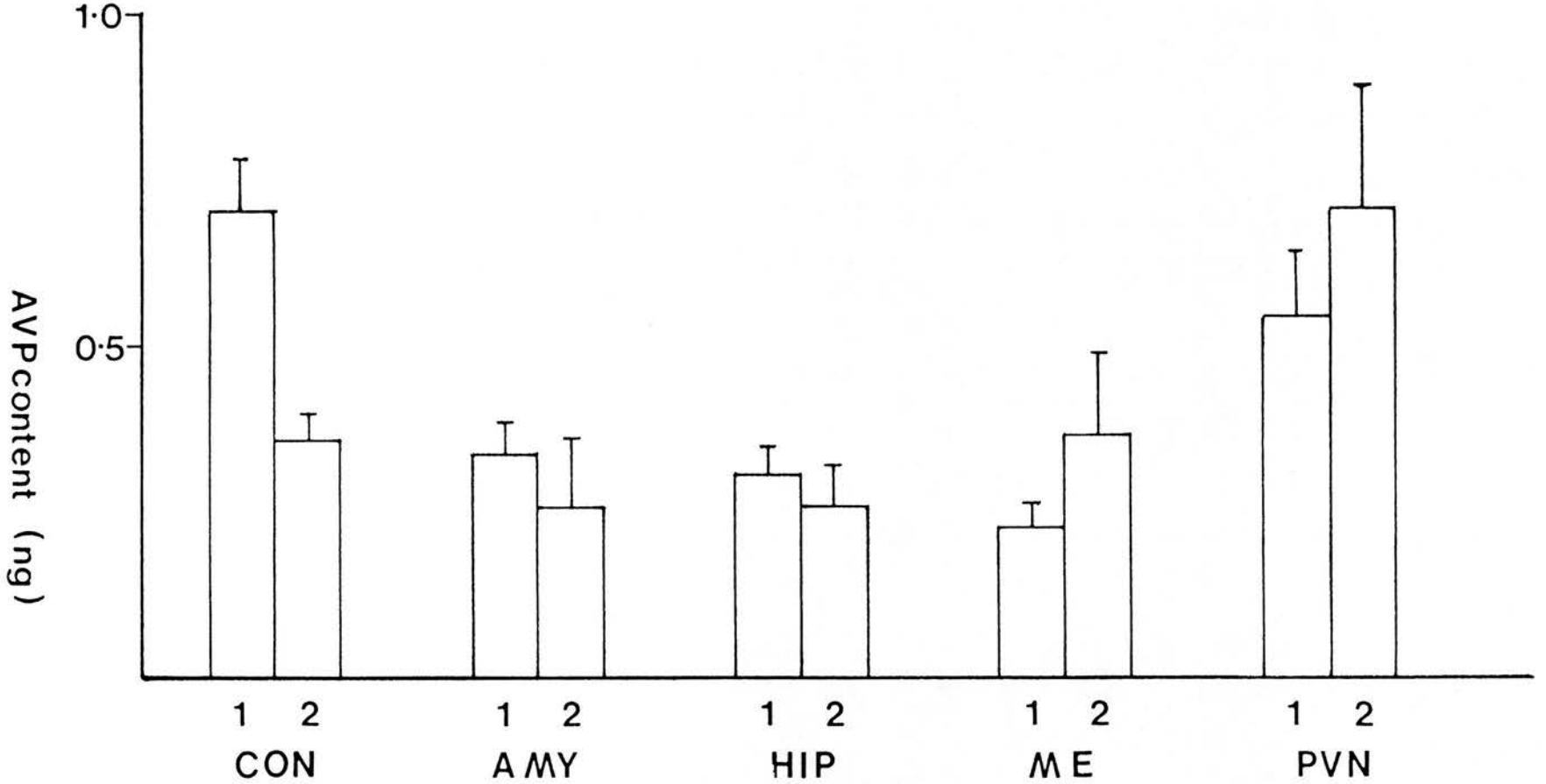
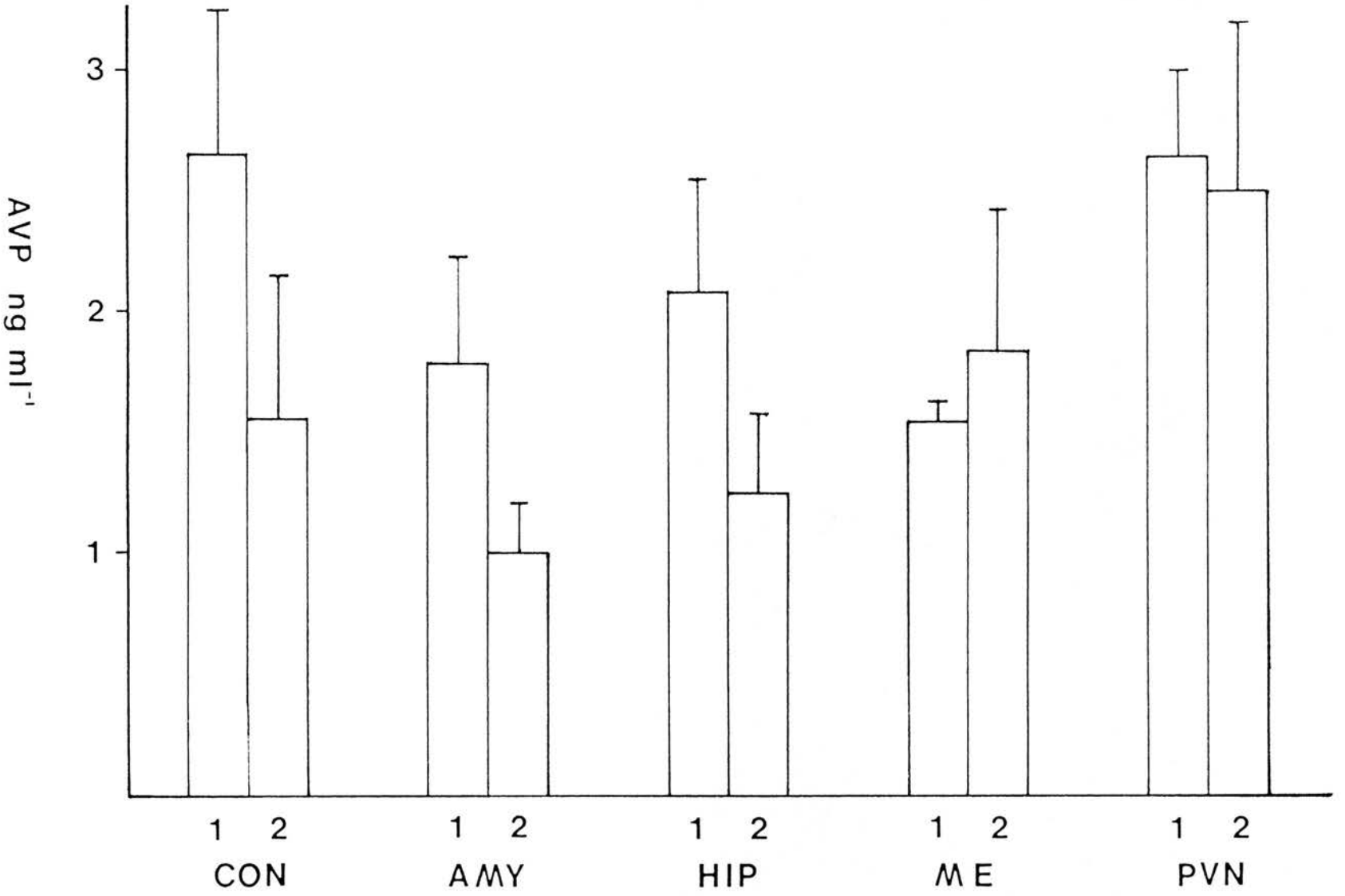

1·0
0·5
AVP content (ng)
1 2
CON
1 2
AMY
1 2
HIP
1 2
ME
1 2
PVN

Figure 7.6 Effect of electrical stimulation on AVP concentration in hypophysial portal plasma

Mean (± S.E.M.) concentration (ng ml^{-1}) of vasopressin (AVP) in hypophysial portal plasma from female Wistar rats with electrodes implanted in the amygdala (AMY; n = 6), hippocampus (HIP; n = 8), median eminence (M.E.; n = 8) and paraventricular nuclei (PVN; n = 8). Hypophysial portal blood was collected during two consecutive 30 min periods (1 and 2) with electrical stimulation applied during the second 30 min collection period. Control rats (CON; n = 6) had electrodes implanted in the amygdala, but no stimulation was applied.


AVP ng ml-1
3
2
1
1 2
CON
1 2
AMY
1 2
HIP
1 2
ME
1 2
PVN

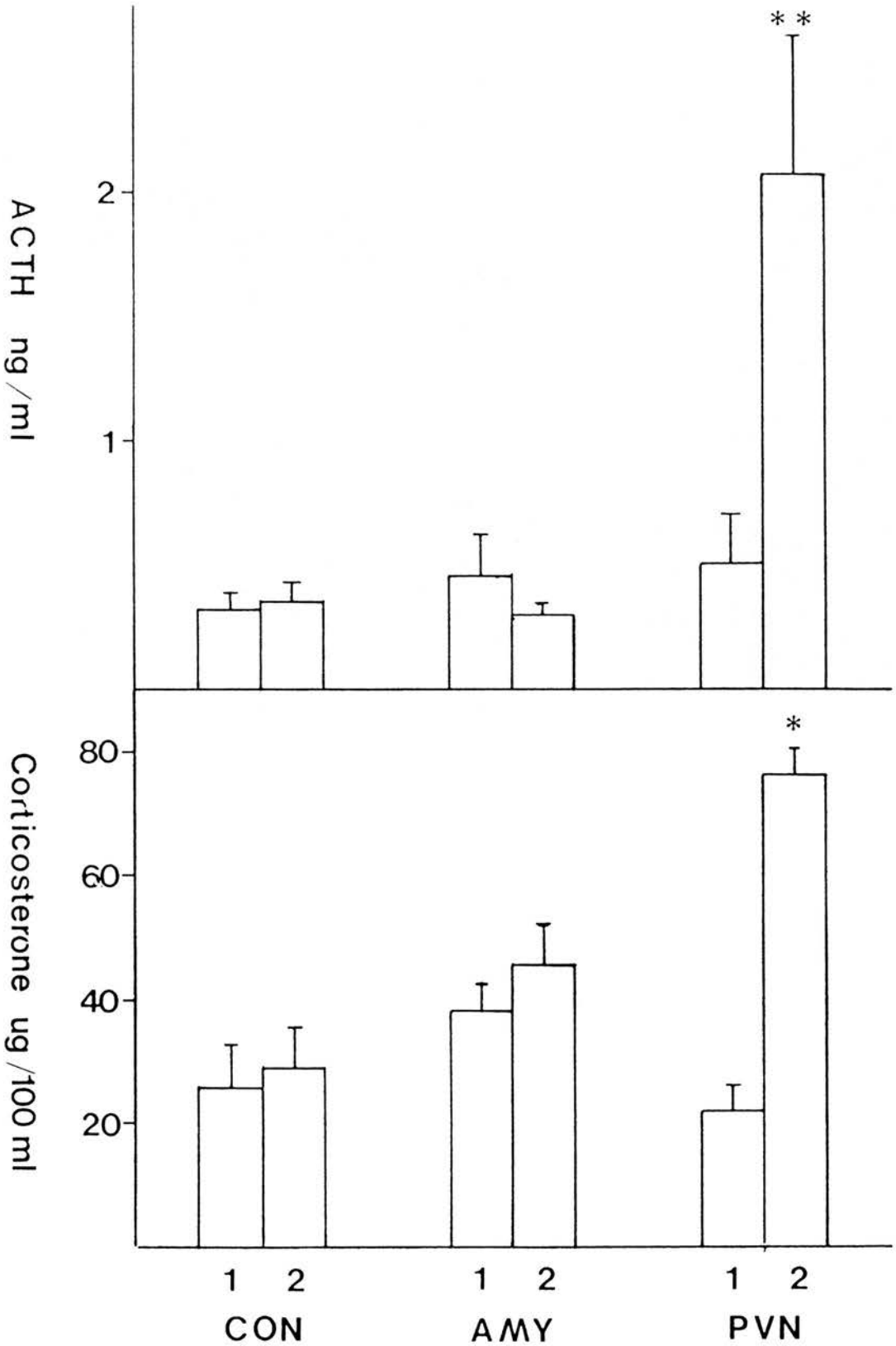
the external jugular vein. In the control animals, electrodes were implanted in the amygdala but no stimulation was applied. Thirty minutes of sham stimulation did not alter plasma ACTH or corticosterone concentrations (Fig. 7.7). Stimulation of the amygdala for 30 min did not significantly alter plasma ACTH or corticosterone concentrations (Fig. 7.7). In contrast, stimulation of the PVN in all animals significantly increased both ACTH and corticosterone concentrations 4-fold relative to that of the concentrations in plasma before application of the stimulus (Fig. 7.7).

7.4 DISCUSSION

The present results demonstrate that stimulation of the ventral hippocampus and amygdala inhibits the release of CRH into hypophysial portal blood. However, changes in CRH release on stimulation of the amygdala did not correlate with the peripheral plasma concentrations of ACTH and corticosterone observed after stimulation of the amygdala in rats in which the pituitary stalk was intact. As expected, stimulation of the PVN resulted in a 2- to 3-fold increase in CRH release into portal blood, and this correlated with a 4-fold increase in the peripheral plasma concentrations of ACTH and corticosterone produced by PVN stimulation in rats in which the pituitary stalk was intact. Stimulation of the median eminence gave variable responses with an overall increase in CRH release which was not significant. Sham electrical stimulation did not affect CRH release into portal blood, or ACTH and corticosterone concentration in peripheral blood. There was no effect of stimulation on the release of AVP into portal blood.

Figure 7.7 Effect of electrical stimulation in the intact rat on ACTH and corticosterone concentrations in peripheral plasma

Mean (± S.E.M.; n = 6) concentrations of ACTH (ng ml^{-1}) and corticosterone (µg/100ml) in peripheral plasma from female rats with electrode implants before (1) and after (2) 30 min of electrical stimulation trains (30s on, 30s off) of biphasic rectangular pulses (50Hz, 1mA peak to peak, 1ms duration). Electrodes were implanted in the amygdala (AMY) and paraventricular nuclei (PVN), control animals (CON) had electrodes implanted in the amygdala but no stimulation was applied. Blood samples were withdrawn from the external jugular vein immediately before and after the 30 min period of stimulation. *$p < 0.001$, ** $p < 0.01$ when compared with plasma concentrations before stimulation (paired t-test).


ACTH ng/ml
2
1
**
Corticosterone ug/100 ml
80
60
40
20
*
1 2
CON
1 2
AMY
1 2
PVN

Numerous studies have shown that the hippocampus modifies adrenocortical activity. Stimulation and lesion studies have demonstrated that the effect of the hippocampus is mainly inhibitory (Casady & Taylor, 1976; Mangili et al., 1966; Knigge, 1961; Feldman & Conforti, 1980; Dunn & Orr, 1984) and the present findings show that this inhibition is mediated by a decrease in release of CRH into portal blood. However, Dunn & Orr (1984) have demonstrated an increase in the plasma corticosterone concentrations following stimulation of the CA1 region of the hippocampus; stimulation of other regions of the hippocampus decreased corticosterone concentrations. The present results are consistent with the findings of Dunn & Orr (1984), as in the present study, the stimulating electrodes were positioned in the ventral hippocampus, while the CA1 region is concentrated mainly in the dorsal hippocampus. Dorsal hippocampectomy significantly reduced the degree of feedback effect of dexamethasone on the adrenocortical response to ether stress while ventral hippocampectomy had no such effect (Feldman & Conforti, 1980). Glucocorticoid receptors are also present at a higher density in the dorsal hippocampus (Fuxe et al., 1985), suggesting that the dorsal hippocampal region participates in the feedback mechanism of glucocorticoids while the ventral region is involved in mediation of other adrenocortical responses, such as those that follow somatosensory stimulation (Conforti & Feldman, 1976).

Stimulation of the amygdala in the anaesthetised intact rat resulted in a slight but not significant decrease in ACTH concentrations in peripheral blood.

The effect of stimulation of

the amygdala in the present study differs from that in the conscious rat (Carrillo & Dunn, 1977) and conscious monkey (Mason, 1959; Frankel et al., 1978) in which stimulation of the amygdala increased plasma corticosterone concentration. Amygdaloid stimulation was found to be ineffective in increasing corticosterone concentrations in rats anaesthetised with pentobarbitone in the study by Carrillo & Dunn (1977) but stimulation of the amygdala did increase corticosterone concentrations in the study by Redgate & Fahringer (1973). The discrepancies in the result of amygdaloid stimulation on corticosterone concentrations may be due, at least in part, to the fact that different regions of the amygdala are differentially involved in adrenocortical function (Dunn & Whitener, 1986) . Stimulation of the ventral and lateral amygdala decreased plasma corticosterone concentrations in rats while stimulation of the medial, basomedial and posterocorticomedial amygdala increased plasma corticosterone concentrations. Positioning of the electrodes in the amygdala in the present study would result in all regions of the amygdala being stimulated and the present results, therefore, suggest that the amygdala exerts a predominantly inhibitory effect on CRH release.

For the sake of clarity, only the sites of stimulation close to the PVN have been included in the statistical analysis of the changes in CRH release due to electrical stimulation. Jamieson & Fink (1976) measured the spread of the square wave stimulus used in the present study by the milk ejection response to stimulation of the hypothalamo-hypophysial tract in a lactating rat. The stimulatory effect was found to 'spread' over a distance of no more than 1.5mm from the electrode tip. In the present study,

stimulation through electrodes positioned within 0.2mm of the PVN result in increased release of CRH, whereas stimulation of the PVN region through electrodes removed by more than 1.5mm from the PVN did not stimulate release of CRH into portal blood. Stimulation of the PVN increased the release of CRH into portal blood 4-fold and in the intact animal, significantly increased ACTH and corticosterone concentrations in peripheral blood. The ability of electrical stimulation of the hypothalamus to increase ACTH and corticosterone concentrations in plasma is in agreement with the findings of Mason (1959) and Frankel et al. (1978) in the conscious monkey and with Dornherst et al. (1986) in the anaesthetised cat.

The present and other findings suggest that the projection from the PVN to the external layer of the median eminence mediates the release of ACTH. Therefore, it should be possible to stimulate the release of CRH and AVP by stimulation of the median eminence. The fact that this was not the case in the present study cannot be due to maximal release as PVN stimulation increased the release of CRH into portal blood. The lack of effect of electrical stimulation of the median eminence was surprising as the parameters used for stimulation in the present study have been shown previously to cause increased release of LHRH, SS and $SS28_{(1-12)}$ into portal blood (Chiappa et al., 1977; Millar et al., 1983; Sheward et al., 1984). Furthermore, AVP and CRH release from the median eminence can be stimulated <u>in vitro</u> by depolarising concentrations of potassium (Holmes et al., 1986) and by low (10^{-10}M) concentrations of β-endorphin (Buckingham, 1986). However, higher doses of β-endorphin reduced not only spontaneous release of CRH but also the secretion elicited by other neurotransmitters. Stimulation of the

median eminence in vivo would also stimulate adjacent areas of the hypothalamus such as the arcuate nucleus. Indeed, Makara et al. (1972) have demonstrated antidromic invasion of hypothalamic neurones outside the supraoptic nucleus and PVN, (e.g. arcuate and ventromedial nuclei) following stimulation of the median eminence. The arcuate nucleus is the source of β-endorphin in portal blood (Wardlaw et al., 1982; Wehrenberg et al., 1982; Sarkar & Yen, 1985) and, therefore, one possible explanation of the fact that median eminence stimulation did not produce an increased release of CRH is that stimulation of the median eminence also releases β-endorphin from the arcuate nucleus which acts to inhibit CRH release. Renaud (1978) has demonstrated recurrent inhibition in parvocellular neurosecretory neurones following stimulation of the median eminence. Renaud suggested that neurosecretory neurones not only have terminals in the median eminence but also produce axon collaterals which form part of an inhibitory feedback pathway to the parent neurone either directly by a synapse on the neurone of origin or indirectly through a transynaptic excitation of local interneurones whose axons then terminate on the neurosecretory cell. Any of these pathways could account for the fact that median eminence stimulation produced only a small increase in CRH release compared with that produced by stimulation of the PVN.

In conclusion, electrical stimulation of the amygdala and hippocampus have an overall inhibitory effect on the release of CRH into hypophysial portal blood but this did not correlate with a decrease in the release of ACTH or corticosterone into peripheral blood. Facilitation of ACTH release by PVN stimulation appears to be a direct result of increased release of CRH into portal blood.

However, in the physiological situation, stimulation of ACTH release by AVP cannot be excluded. From the results of the present study, the PVN appears to be the major central node controlling the release of ACTH from the anterior pituitary gland. The different responses to stimulation in the release of CRH and AVP is also indicative of differential regulation of release of these ACTH secretogogues from the brain.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The techniques of hypophysial portal blood collection and radioimmunoassay were used to examine the relative importance and the relationships between corticotrophin releasing hormone (CRH), vasopressin (AVP) and oxytocin (OT) in stimulating the secretion of adrenocorticotrophin (ACTH) in vivo. Factors affecting synthesis of proopiomelanocortin (POMC), the precursor for ACTH, were examined by measuring the content of POMC mRNA in the anterior pituitary gland by Northern blot analysis.

It is well established that the secretion and synthesis of ACTH is modulated by the negative feedback action of glucocorticoids. Therefore, to examine the mechanism of negative feedback of glucocorticoids, the effect of adrenalectomy on the release of CRH, AVP and OT into portal blood, the pituitary responsiveness to CRH and on the content of POMC mRNA in the anterior pituitary gland was examined. Removal of the negative feedback of glucocorticoids by adrenalectomy increased the content of POMC mRNA in the anterior pituitary gland and also increased the secretion of ACTH into peripheral plasma. The increase in the release and synthesis of ACTH after adrenalectomy is a direct result of the significant increase observed in the release of CRH and AVP into portal blood after adrenalectomy. Hypothalamic content of CRH mRNA (Jungami et al., 1985) and AVP mRNA (Davies et al., 1986) have been shown to increase after adrenalectomy suggesting that the increase observed in the present study in the release of CRH and AVP into portal blood is due, at least in part, to the increased synthesis of the precursors for CRH and AVP. Replacement of glucocorticoids for an intermediate time period in adrenalectomised rats decreased the release of AVP but not CRH into portal blood when compared with

untreated adrenalectomised rats. These results suggest there is differential regulation of CRH and AVP release by glucocorticoids at the intermediate level of feedback. Differential regulation of CRH and AVP has also been observed in response to haemorrhage (Plotsky et al., 1985b) and in response to insulin-induced hypoglycemia (Plotsky et al., 1985c).

Although the release of CRH into portal blood is unaffected by restoration of negative feedback for 3h, the effectiveness of CRH in stimulating ACTH secretion is drastically reduced by a reduction in the responsiveness of the pituitary gland to CRH. These findings suggest that glucocorticoids exert their action on the hypothalamus and on the pituitary gland to control ACTH secretion.

A variety of stressors are well known to cause release of ACTH and glucocorticoids into peripheral plasma. To determine whether a short-term increase in the demand for ACTH secretion would be accompanied by an increase in the synthesis of ACTH, the content of POMC mRNA in the anterior pituitary was measured at various time points after a 5 min period of ether stress. Ether stress immediately increased the peripheral plasma concentrations of ACTH and corticosterone 10- and 3-fold respectively, 6h later, ACTH concentrations were 5-fold higher than in the control animals. Twenty-four hours after the stress, plasma concentrations of ACTH and corticosterone were similar to those in control animals. After 6h, POMC mRNA content in the anterior pituitary gland was 2-fold greater than in control animals, suggesting that the biosynthetic and secretory activities of the anterior pituitary gland are coupled to enable the animal to respond to stress with a dramatic increase in ACTH release.

The neural apparatus which regulates adrenocortical function is generally held to comprise of two components: a hypothalamic component and a limbic-midbrain component. The effect of electrical stimulation of the various brain areas implicated in the regulation of adrenocortical function on the release of CRH and AVP into hypophysial portal blood was examined. Changes in the release of these peptides were then compared with the effect of electrical stimulation on the synthesis of ACTH and on ACTH and corticosterone concentrations in peripheral plasma in animals in which the pituitary stalk was intact. The release of CRH into portal blood was significantly increased during stimulation of the PVN and this correlated with a significant increase in ACTH and corticosterone in peripheral plasma following 30 min of PVN stimulation. However, POMC mRNA content in the anterior pituitary gland was unchanged 6h after electrical stimulation of the PVN suggesting that the increase in CRH release was not sufficient to increase transcription of the POMC gene. Stimulation of the amygdala and the hippocampus reduced the release of CRH into portal blood. However, the decrease in CRH release following stimulation of the amygdala did not correlate with changes in ACTH and corticosterone concentrations in animals in which the pituitary stalk was intact and there was no change in POMC mRNA content in the anterior pituitary gland. Stimulation of the median eminence did not significantly increase the release of CRH. Release of AVP into portal blood was not altered by stimulation of any of the brain areas tested. The parvocellular region of the PVN contains AVP and CRH neurones that terminate on hypophysial portal blood vessels in the median eminence and, therefore, it could be expected that stimulation of both the PVN and the median eminence

would increase the release of CRH and AVP into portal blood. However, stimulation of the median eminence *in vivo* would also undoubtedly stimulate adjacent areas of the hypothalamus such as the arcuate nucleus, which is the source of β-endorphin in portal blood (Sarkar & Yen, 1985). Therefore, it is possible that β-endorphin is also released during stimulation of the median eminence with the result of inhibiting the release of CRH and AVP into portal blood. Renaud (1978) has demonstrated recurrent inhibition in parvocellular neurosecretory neurones following median eminence stimulation. Renaud suggested that neurosecretory neurones produce axon collaterals which form part of an inhibitory feedback pathway to the parent neurone either directly or indirectly through local interneurones, whose axons then terminate on the neurosecretory cell. These pathways could account for the small increase observed in CRH release into portal blood during stimulation of the median eminence. These results suggest that the PVN is the major central mode for regulating adrenocortical function.

The possibility that release of opioid peptides during median eminence stimulation could inhibit the release of AVP and OT was examined as it had proved impossible, by electrical stimulation, to increase the release of these peptides from the median eminence *in vivo*. Infusion with naloxone 1h before and during electrical stimulation of the median eminence did not affect the release of AVP and OT into portal blood. This was surprising, as there are many reports on the inhibition of OT release and a few reports on inhibition of AVP release from the neurophysis by opioid peptides. An inhibitory role for opioid peptides in the control of release of AVP and OT cannot be ruled out. The inhibitory and stimulatory

actions of β-endorphin on CRF release have been shown by Buckingham & Cooper (1986) to be mediated by different opioid receptor types. The δ receptors thought to be involved in the inhibitory action of β-endorphin are not readily antagonised by naloxone. Therefore, in the present study, the lack of effect of naloxone cannot rule out the possibility that opioid peptides prevent an increased release of AVP and OT into hypophysial portal blood in response to electrical stimulation.

The first, and major, endocrine abnormality to be described in the homozygous Brattleboro rat was the lack of AVP which results in diabetes insipidus. Schmale & Richter (1984) have shown that there is a single base deletion in the protein coding region of the AVP gene which results in an 'out of frame' reading sequence and, therefore, impedes AVP mRNA translation. In spite of this, AVP immunoreactivity has been shown in the ovary, adrenal gland and anterior pituitary gland of the homozygous Brattleboro rat. In the present study, AVP immunoreactivity was found in hypophysial portal blood from homozygous Brattleboro rats at concentrations of approximately 50% of those found in control Long Evans rats. Portal blood from homozygous Brattleboro rats was further analysed by high performance liquid chromatography and the 'AVP' was found to be identical to authentic AVP. Hypothalamic AVP was undetectable in homozygous Brattleboro rats and this could be due to the dilution factor used in the assay being too high or it could reflect a highly efficient processing of AVP from the hypothalamus, at the level of the external layer of the median eminence, into portal blood. These results combined with the evidence of Mezey et al. (1986) that homozygous Brattleboro rats make small amounts of the normal AVP

precursor in the PVN and in the posterior pituitary suggests that the defect in the homozygous Brattleboro rat is not the same as the defect reported by Schmale & Richter (1984). Alternatively, there may be more than one AVP gene and they can be processed in a tissue-specific manner which results in a relatively high concentration of AVP in portal blood but very little AVP in the posterior pituitary gland.

There was no significant difference in CRH or OT concentrations in portal blood between control Long Evans and homozygous or heterozygous Brattleboro rats. ACTH and corticosterone concentrations in peripheral blood were lower in Brattleboro rats compared with Long Evans rats, but the difference was only significant in the heterozygous Brattleboro rat. The slight reduction in ACTH and corticosterone in the homozygous Brattleboro rat may be due to the reduced secretion of AVP into portal blood. However, given the synergistic relationship of AVP with CRH, a greater deficit might be expected in ACTH secretion in the Brattleboro rat. In both genotypes of Brattleboro rat, POMC mRNA in the anterior pituitary gland is 2-fold more abundant than in control Long Evans rats. Therefore, it may be possible that the adrenocortical feedback system has a different set point in Brattleboro rats, the reduced concentration of corticosterone having less feedback effect on the anterior pituitary gland, thereby increasing POMC mRNA synthesis and ACTH release.

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APPENDIX I

Buffers

Phosphate-EDTA pH 7.4:

63mM $Na_2H\ PO_4$

13mM Na_2 EDTA

0.02% NaN_3

250 KIU ml^{-1} Trasylol

0.01M PBS pH 7.4:

140mM NaCl

2.5mM $NaH_2\ PO_4\ 2H_2O$

8mM $Na_2H\ PO_4$

0.25mM Thimerosal

PBS/NRS pH 7.4:

PBS containing

0.05M EDTA

0.5% NRS

1.0% BSA

ACTH RIA Buffer pH 7.4:

63mM $Na_2H\ PO_4$

13mM Na_2 EDTA

0.02% NaN_3

RIA Buffer for AVP (2.7):

0.1M Phosphate

0.5M NaCl

0.1% BSA

0.01% NaN_3

APPENDIX II

Detection of Specific Nucleic Acid Sequences within Heterogeneous Populations of Molecules by Gel Transfer Hybridisation

A type of gel transfer hybridisation has been developed to analyse the distribution of specific RNA sequences within a population of electrophoretically fractionated RNA molecules. A solid-phase support is used to bind and immobilise the RNA in the position to which it has migrated on a gel. This technique is described as 'Northern' blotting. The support originally used is paper derivitised with diazobenzyl-oxymethyl groups to which single-stranded nucleic acid will bind covalently. It has subsequently been shown that nitrocellulose sheets will bind RNA that is fully denatured. This is also important to achieve a linear inverse relationship between the mobility of an RNA molecule and its molecular length. The gels are therefore run in the presence of methylmercury, glyoxyl or formaldehyde as denaturing agents. Following its transfer to diazobenzyl-oxymethyl paper or nitrocellulose, the immobilized RNA can be hybridised with a radiolabelled DNA probe. In order to prevent non-specific binding of the radiolabelled DNA probe, the diazobenzyl-oxymethyl paper or nitrocellulose is incubated for 2-4 h in a prehybridisation buffer which contains denature salmon sperm DNA. Hybridisation of the DNA probe to RNA immobilised on nitrocellulose is carried out for 16 h at 42°C in a buffer which contains 50% formamide and 5-10% dextran sulphate. Hybridisation reactions are faster in an aqueous solution at 68°C but the high temperature presents problems of evaporation and damage to the nitrocellulose. In the presence of dextran

sulphate, the rate of association of nucleic acids is accelerated because the nucleic acids are excluded from the volume of the solution occupied by the polymer. However, the use of dextran sulphate is not always necessary to detect sequences of interest. It is also difficult to handle because of its viscosity and it can sometimes lead to high backgrounds. The DNA probe is added to the nitrocellulose in as small a volume of hybridisation buffer as possible. The kinetics of nucleic acid reassociation are faster and the amount of probe may be reduced. However, it is essential that sufficient liquid be present for the nitrocellulose to remain at all times covered by a film of the hybridisation solution. Excess radiolabelled DNA probe is then washed off the nitrocellulose and the nitrocellulose autoradiographed with the aid of intensifier screens.

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